1990

Ultrastructural and Invasive Characteristics of Murine B16a Melanoma Cells: An in Vitro and in Vivo Study

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ULTRASTRUCTURAL AND INVASIVE CHARACTERISTICS OF MURINE B16a MELANOMA CELLS: AN IN VITRO AND IN VIVO STUDY

BY

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May

1990

DEDICATION

To Mom, Dad, and the Kids

ACKNOWLEGDMENTS

I wish to thank my advisor, Dr. Bruce Persky, for his help, sense of humor, and his drive against all odds. This work could not come to light without his guidance and attention to detail. I would like to thank Dr. John Clancy, Dr. Gary Schneider, Dr. Richard Schultz, and Dr. Andrew Bajkowski for their advice and critical review of this work. I would also like to thank Dr. John McNulty and Linda Fox for their assistance with the electron microscopic studies and image analysis. A special thanks goes to Dr. Simone Silberman without whom many more mice would have died during the in vivo studies. Of course, a special thanks goes to Ken, Mike and Bob. The world hasn't seen much of this foursome lately, hence world peace appears to be at hand. In addition, words do not do justice to the help and encouragement, Val has given me throughout. Lastly, much appreciation and love goes to my family. Their moral support was invaluable at all times.

VITA

The author, Terence W. McGarvey, is the oldest son of Terence and Mary McGarvey. He was born on September 8, 1960 in Hollis, New York.

His secondary education was completed at H.F. Carey High School in Franklin Square, New York in June 1978. He attended Hofstra University, Hempstead, New York and graduated in May, 1982 with a Bachelors of Arts in Biology (minor in Chemistry). He attended the C.W. Post campus of Long Island University, Greenvale, New York and graduated in May, 1985 with a Masters of Science in Bioeducation. In August of 1985, he entered the Department of Anatomy of the Graduate School at Loyola University of Chicago. While at Loyola, he received a Basic Science Fellowship for 1985-89 and a Schmitt Fellowship for 1989-90. He taught one year of gross anatomy, one year of histology, and three years of neuroscience in the medical school. In 1989, he received a Sigma Xi grant. He is a member of the American Society for Cell Biology, the Society of Sigma Xi, and the American Association of Anatomists. He was an active member of the Graduate Student Council and held the offices of Graduate Student Representative and Treasurer.

After completion of his doctorate, he will begin a post-doctoral fellowship in the Department of Pharmacology at Fox Chase Cancer Center, Philadelphia, Pennsylvania under the supervision of Dr. Mark E. Stearns.

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CHAPTER I

INTRODUCTION

This investigation examines the effect of two antitumor drugs, all trans-retinoic acid (RA) and butyric acid (BA), on the metastatic properties of murine B16a melanoma cells. The B16a cell line is the murine counterpart to human melanoma which is the most lethal form of skin cancer. Accepted forms of chemotherapy have been found to be ineffective in controlling metastatic melanoma (McClay and Mastrangelo, 1988).

Vitamin A (retinol) and metabolites (such as retinoic acid) are necessary for the visual system, normal development and tissue differentiation of an organism. Evidence is accumulating for retinoids in chemoprevention and therapeutic control of cancer (Lippman et al., 1988). Butyric acid (and its sodium salt) is a natural four carbon fatty acid normally present in lipids. Much less is known about BA than RA in the normal development of an organism. However, a role for BA in the control of neoplastic formation has been found (Kruh, 1982).

Retinoic acid and BA warrant investigation for two main reasons. First, RA and BA can be less toxic and less mutagenic to the host than conventional chemotherapeutic drugs when administered at cytostatic concentrations, i.e. the concentration of a drug that inhibits cellular proliferation without causing cell death. Second, the effects of RA and BA on the individual steps of the metastatic cascade are undefined.

Most of the deaths due to cancer are a result of the appearance of neoplasms in parts of the body removed from the primary lesion. The metastatic cascade is a complex series of steps involving: (1) primary growth and progression of the primary tumor, (2) intravasation, i.e. the

escape of tumor cells into the lymphatics and blood vessels, (3) dissemination of cells through the lymphatics and blood vessels, (4) arrest of cells within the walls of small vessels, (5) extravasation, the escape of tumor cells from small vessels and into surrounding tissues, and (5) cell proliferation at the new secondary site. Important host tumor cell interactions can take place at any point of the cascade. Experimental evidence suggests that only a select few tumor cells are capable of completing this sequence of events (Poste et al., 1980).

A tumor cell must invade various tissue barriers, which include the extracellular matrices and basement membranes, for metastasis to take place. Migration, defined here as the ability of a cell to locomote and deform, is an important part of the invasion process.

The cytoskeleton is a network of proteineous filaments found throughout the cytoplasm of all eukaryotic cells. The cytoskeleton includes microtubules (25 nm in diameter), intermediate filaments (10-15 nm in diameter) and microfilaments (5-7 nm in diameter). The cytoskeleton has many different functions essential for the normal functioning of the cell including cellular migration. However, the importance of the cytoskeleton in the metastatic process is unclear.

Five parameters of the metastatic cascade that I am investigating are: (1) determination of the cytostatic and cytotoxic concentrations of RA and BA for the B16a cell line, (2) quantification of in vitro migration, (3) quantification of in vitro invasion, (4) quantification of in vivo experimental metastasis, and (5) cytoskeletal morphology. The experimental metastasis assay is the inoculation of tumor cells into

the murine lateral tail vein followed by extravasation of tumor cells in the lungs. This project emphasizes the evaluation of *in vitro* experiments in light of *in vivo* assays, and stresses the importance of testing potential antitumor agents by multiple parameters. This project is novel in that it will lead to further understanding of the multistep process of metastasis by *in vitro* methods following RA and BA treatment. In this study, evidence for a role for RA and BA in the treatment of metastasis was found. In addition, both drugs present interesting model systems for evaluating the mechanisms behind tumor cell migration and invasion.

CHAPTER II

REVIEW OF LITERATURE

Cancer and Metastasis

The second leading cause of death in the United States is cancer. Most of these deaths are a consequence of metastasis or complications as a result of the treatment of the metastasis. Of the million new cases of cancer each year, two thirds of the individuals will develop and die of metastasis (American Cancer Society Facts and Figures, 1982). Metastasis is defined as the appearance of neoplasms in parts of the body remote from the primary tumor. Metastasis is a complex series of events starting with initiation, growth, and progression (diversification) of a primary tumor. Some tumor cells have the inherant ability to invade local tissues and enter the venous and lymphatic vessels (intravasation). Single tumor cells or tumor emboli may travel within these vessels to distant sites and then arrest in capillaries. Surviving tumor cells may exit the capillaries (extravasation) and establish colonies. Fortunately, progress has been made in the last two decades towards understanding the metastatic process.

Metastasis and Invasion

The extracellular matrix is a major barrier to a tumor cell in the metastatic cascade. A tumor cell must invade the extracellular matrix at multiple points during metastasis. The extracellular matrix is a complex meshwork of collagens and elastin embedded with a ground substance consisting of glycoproteins (for example: laminin and fibronectin) and various proteoglycans. Some of the functions of the extracellular matrix include supporting tissues, influencing cell attachment, mitogenesis, morphogenesis, cellular differentiation and

determining tissue architecture. A specialized portion of the extracellular matrix is the basement membrane (the electron microscopic term is basal lamina). Tumor cells must cross basement membranes in order to invade most organ parenchyma, and to intravasate and extravasate. Liotta has proposed a three-step hypothesis to describe tumor cell invasion. The tumor cell must first attach to the matrix. Subsequently, the tumor cell secretes various proteolytic enzymes which locally degrade the matrix. Lastly, the tumor cell must migrate into the space created by proteolytic digestion (Liotta et al., 1983). Many investigators have chosen to examine the interaction of tumor cells with the extracellular matrix as part of a reductionist strategy in order to begin to understand the complex nature of the metastatic process.

Invasion Assays

Various in vitro invasion assays have been used to study the mechanisms of invasion (Liotta et al., 1982) as well as evaluating various drugs for anti-invasive properties (Welch et al., 1989). These methods include: (1) organ culture with such diverse targets of invasion as embryonic heart muscle (Mareel et al., 1979), lung tissue (Paweletz et al., 1986), kidney (Castro and Cass, 1974), murine mesentery (Lee et al., 1988), and brain tissue microspheres (Waller et al., 1986), (2) cellular monolayers of fibroblasts (Chew et al., 1988), endothelial cells (Waller et al., 1986; Nakajima et al., 1987), and endothelial cell-fibroblast gels (Schor et al., 1985), (3) the chick chorioallantoic membrane (CAM) (Scher et al., 1976), (4) the human amnion basement membrane (HABM) (Liotta et al., 1980; Schleich et al.,

1981), (5) the lens capsule (Starkey et al., 1984a; 1984b) and most recently (6) reconstituted basement membrane (Terranova et al., 1986; Albini et al., 1987). Each of these protocols has their proponents as well as detractors. What is required is an in vitro assay that is repeatable, easy to perform and quantifiable as well as one that closely mimics the clinical situation (Poste, 1982).

Malignant Melanoma

The most lethal form of skin cancer is malignant melanoma. It is estimated that in 1990, twenty-two percent of the patients diagnosed with melanoma will die (CA). Melanoma accounts for only 1% of all cancer, but the incidence of melanoma continues to rise. The five year survival rate of patients presenting a clinically localized disease can be estimated from the level of invasion. Those diagnosed with a distant metastases have less than a ten percent chance of survival (for a review see, Liotta et al., 1987). Human melanoma is refractive to most drug therapies. Dacarbazine (DTIC) is viewed as the treatment of choice for patients that present with metastasis. The overall response (complete and partial) rate has been reported to be 21% with the majority of the responses being partial and of short duration. A complete response is defined as disappearance of the tumor for five years. Phase II clinical trials of multiagent regimens with dacarbizine, cisplatin, 1,3-bis-2 chloroethyl 1-nitrosourea, and tamoxifen had a overall response rate of 52.5% with 7 complete and 14 partial responses (McClay and Mastrangelo, 1988).

Cancer Chemotherapy

Cancer chemotherapy is currently being reevaluated for several

reasons including: (1) drug carcinogenicity to normal tissues (Benedict et al., 1979), (2) enhancement of tumor cell diversity (McMillian and Hart, 1986), and (3) progression of invasive and metastatic capabilities (Nicolson, 1984). Chemotherapeutic drugs have been shown to damage normal tissues and allow surviving tumor cells to metastasize more easily (Nicolson and Custead, 1985). The damage done by antitumor agents to the host may include immunosuppression such as reduced natural killer cell activity (Nolibe and Poupon, 1986), or vascular injury such as damage done to endothelial cells (Nicolson and Custead, 1985). One chemotherapeutic strategy is to use cytostatic agents or so called differentiation agents that are less host-toxic and less mutagenic than conventional chemotherapy. Cytostatic agents may be used as adjuvants between cycles of highly cytotoxic drugs to prevent regrowth and tumor cell diverification (Nicolson and Lotan, 1986).

Differentiation and Malignancy

The relationship between differentiation and malignancy has received a great deal of attention. Certain common tumors in man, such as squamous cell carcinoma of the lung, represent a change in the pathway of differentiation. Normal lung epithelium is columnar and mucous secreting. The term used for abnormal differentiation is transdifferentiation (Wyllie et al., 1981). It also has been observed that as a cancer progresses, the neoplasm becomes more dedifferentiated (anaplastic) (for review, see Freshney, 1985). A general histological progression can be seen in the production of the malignant phenotype. Loss of growth control is a hallmark of benign as well as malignant

tumors, but local invasion (including degradation of local basement membranes) is the hallmark of a malignant tumor.

The normal cell counterpart to malignant melanoma is the melanocyte. Melanocytes are unique cells found in the mucous membranes, the choriod of the eye, the piaarachnoid of the nervous system, and in basal layer of the epidermis. Melanocytes have a phenotype that is distinctive from other cell types. These characteristics include (1) dendritic formation, (2) pigmentation due to production of melanin in specific organelles (melanosomes) and, (3) expression of the specific enzyme tyrosinase. Tyrosinase converts tyrosine to 3,4-dihydroxy-phenylalanine (dopa) and dopa to dopaquinone. Melanocytes are derived from cells migrating out of the neural crest. Neural crest cells give rise to a number of other differentiated cell types including neurons and supporting cells of the dorsal root and autonomic ganglia and mesenchyme of the head. The question remains at what stage or stages of melanocyte differentation are targets for malignant transformation. A possible model for understanding malignant transformation and progression in human melanoma is benign focal proliferation of melanocytes in melanocytic nevi.

A panel of 25 different human melanomas have been characterized on the basis of early, intermediate, or late stages of melanocyte differentiation (Houghton et al., 1982). These melanomas were classified into three groups (early, intermediate and late stage) based on phenotypic characteristics including pigmentation, morphology, tyrosinase activity and cell surface antigens. Four different sets of melanoma surface antigens were categorized on the basis of reactivity

of mouse monoclonal antibodies with fetal, newborn, and adult melanocytes. Early stage melanomas were characterized by an epitheloid morphology, lack of pigmentation, lack of tyrosinase expression, and early marker antigens. Early marker antigens are not expressed by fetal, newborn or adult melanocytes. Intermediate melanomas had a bipolar shape and expressed antigens also found on the surface of fetal or newborn melanocytes. Late stage melanomas had extensive dendritic formation, heavy pigmentation, high tyrosinase activity, and expressed antigens found on adult melanocytes (Houghton et al., 1982). No correlation has been found for human melanoma cell lines between melanogenesis and tumor latency, size, or frequency of metastases (Aubert et al., 1980). However, various cell surface antigens have been linked to melanoma progression. Antigens K.1.2. and HLA-A,B,C have been found to be decreased in the course of local and systemic melanoma spread, while antigens HLA-DR, A.1.43, and A.10.33 were increased in proportion to invasiveness and metastatic properties (Brocker et al., 1985).

Murine Melanoma

The B16 melanoma cell line is a murine counterpart of human melanoma. The B16 cell line was adapted to tissue culture by Fidler (1970) from a transplantable tumor originating spontaneously in the C57BL/6J mouse in 1954. The B16a (amelanotic) cell line is highly invasive and spontaneously metastatic and has been fully characterized by K. Honn (Crissman et al., 1983). The phenotype of the B16a melanoma cell line does not seem to fit into one of the general melanoma phenotypes, but instead has dendritic formation without pigmentation.

Possible differentiation antigens have been identified in the B16 cell line. The Met-72 antigen has been correlated to increased metastasis in vivo (Kimura and Xiang, 1986). The Norm-1 and Norm-2 antigens have been linked to increased proliferation. Antibodies to Norm-1 and Norm-2 decrease proliferation and induce changes in morphology of B16 cells (Vollmers et al., 1985). Low serum content in media, high cell density or the administration of transforming growth factor beta (TGF-beta) decreases Norm-2 antigen presentation (Wieland et al., 1986).

Differentiation Agents and Melanoma

Tumors of ectodermal, neuroectodermal, mesodermal and endodermal origins have been shown to undergo some form of differentiation in response to various inducers (for review see Freshney, 1985). Melanoma is an interesting model system for differentiation. Human melanomas have a varied response to inducers in that differentiation can either be accompanied by a decrease or increase in tumorgenicity in nude mice (Aubert et al., 1980). Tryrosinase activity, changes in proliferation, modifications in tumor cell phenotype, as well as alterations in cell surface markers, vary from cell line to cell line in response to a number of physiological inducers. Several hormones, including melanocyte-stimulating hormone (MSH) (Fuller and Meyskens, 1981), corticotropic hormones (Abramowitz and Chavin, 1978) and prostaglandin A_1 and E_1 (Fuller and Meyskens, 1981; Bregman et al., 1983) have been shown to stimulate tyrosinase activity and melanin production in normal melanocytes as well as melanoma cell lines. Low metastatic B16F1 cells were found to be sensitive to MSH or cAMP. Melanocyte stimulating

hormone or cAMP induced tyrosinase activity and inhibited proliferation in B16F1 cells. However, highly metastatic B16F10 cells were not sensitive to MSH or cAMP (Niles et al., 1978). Melanocyte stimulating hormone and theophylline (an inducer of cAMP) inhibited proliferation, promoted differentiation, and reduced tumorgenicity of murine S91 melanoma cells (Wick, 1981). Prostaglandin derivitives have been shown to inhibit the growth of B16a cells (Honn et al., 1981).

Nonphysiological inducers such as polar solvents can modify the phenotype of melanoma cells in vitro. An inhibitor of polyamine synthesis, 2-difluromethylornithine (DFMO) stimulated melanogenesis and inhibit proliferation of S91 cells (Kapyaho and Janne, 1983). DFMO was shown to inhibit proliferation of B16F1 cells in vitro without an increase in tyrosinase activity (Sunkara et al., 1983). Polar solvents such as dimethyl sulfoxide (DMSO) have been shown to induce growth inhibition and to produce changes in morphology of human melanoma cells (Huberman et al., 1979) and murine B16 cells (Lampugnani et al., 1987). Dexamethasone (a glucocorticoid agonist) was shown to inhibit the proliferation of six different human melanoma cell lines (Osman et al., 1985). Even cytotoxic agents, such as the anthracycline antibiotics, have been shown to inhibit growth and produce irrevisible morphological changes in B16F1 cells after 16 hrs of incubation (Raz, 1982). The roles of butyric acid and the metabolites of vitamin A, 13 cis and all trans-retinoic acid in tumor cell differentiation, will be discussed later.

The effect of differentiation agents on melanomas in vivo has been investigated. Drinking water containing 2% DFMO has been shown to

decrease the tumor weight of B16F1 tumors by 84% in C57BL6 mice (Sunkara et al., 1983), and decrease spontaneous metastasis of B16a cells (Sunkara and Rosenberger, 1987). The mechanism of the inhibition of spontaneous metastasis was believed to be in the intravasation step of the metastatic cascade, since the administration of DFMO did not reduce lung colonization by tail vein inoculation (experimental metastasis) (Sunkara and Rosenberger, 1987). Pretreatment of B16 melanoma cells, on the other hand, with differentiation agents has been shown to decrease tumorgenicity (Nordenberg et al., 1985), but increase experimental metastasis (Bennett et al., 1986; Casali et al., 1988). A 24 hr incubation of B16F1 or B16F10 cells in medium with a higher pH (from 6.9 to 7.2) and containing 10^{-9} M MSH was shown to produce an increase in tyrosinase activity in vitro and experimental metastasis in vivo (Bennett et al., 1985). Experimental metastasis was also shown to be significantly increased by DMSO pretreatment of B16 cells for 4 days (Casali et al., 1988). On the other hand, a delay was observed in the onset of tumor formation of DMSO pretreated B16F10 cells (Nordenberg et al., 1985).

The Retinoids: Function in Normal Development

Vitamin A (retinol) and metabolites are essential for maintainance of the visual system (Bridges, 1984), for normal embryonic development, for differentiation and maintenance of epithelial tissue (for review see Roberts and Sporn, 1984), and for the development and maintenance of the immune system (Dennert, 1984). The major sources of vitamin A in the diet are certain plant carotenoid pigments, such as B-Carotene, and long chain retinyl esters found in animal tissues. B-Carotene and

retinyl esters are converted to retinol by the intestinal mucosa (Goodman and Blaner, 1984). Many investigators believe that retinoic acid is close to the structure of the retinoid that acts within cells to control development (Frolik, 1984). Retinoids at low concentrations are known teratogens and induce major craniofacial deformities in rodent models (Shenefelt, 1972).

The hypothesis that retinoids may suppress carcinogenesis (delaying the preneoplastic progression of carcinogen or radiation-treated cells), induce neoplastic differentiation, inhibit tumor cell proliferation, and suppress various tumor phenotypic properties was not difficult to believe given the role of retinoids in normal development.

Retinoids and Carcinogenesis

The chemopreventitive nature of the retinoids has been shown to be important in inhibiting the promotion phase of chemically induced rodent skin, mammary gland, and urinary bladder carcinogenesis (for review see Moon and Itri, 1984). A link has been observed between a decreased human dietary intake of vitamin A and increased incidence of several carcinomas (for review see Moon and Itri, 1984). Retinoids have also been used to treat preneoplastic conditions including dysplastic nevi (Lippman et al., 1987). Dysplastic nevus is characterized by the presence of pigmented flat papules or macules that frequently undergo transformation to melanoma. Three patients were treated for 10 to 12 weeks with topical all trans-retinoic acid. The dysplastic nevus features underwent major changes to benign nevocellular nevi (Lippman et al., 1987).

Retinoids and Tumor Cell Differentiation

Retinoids have been shown to have direct effects on proliferation and differentiation of tumor cells. Most of the studies have been done on tumor cells in vitro, but some in vivo and clinical studies that have been performed indicate a possible role of retinoids in the treatment of tumors and metastasis. The effect of retinoids on tumor cells seems to depend on the cell line being studied. The effect of retinoic acid on tumor cell differentiation can be divided into two general categories, terminal and reversible differentiation.

Terminal differentiation occurs in murine embryonal carcinoma (Strickland and Mahdavi, 1978; Jetten et al., 1979), human promyelocytic leukemia (Breitman et al., 1980), and rat rhabdomyosarcoma (Gabbert et al., 1988). An intermediate phenotype was induced in retinoic acid-treated F9 teratocarcinoma cells that could differentiate further into either visceral or parietal endoderm depending on culture conditions (Hogen et al., 1981). Differentiation was measured by the induction of plasminogen activator (Strickland and Mahdavi, 1978). Retinoic acid-treated human promyelocytic (HL-60) cells differentiate into functionally and morphologically mature granulocytes (Breitman et al., 1980). Terminal differentiation with exposure to retinoic acid was shown to occur with a rat rhabdomyosarcoma cell line (BA-HAN-1C). There was an increase in the number of terminally differentiated postmitotic myotube-like giant cells and an increase in the creatine kinase activity (Gabbert et al., 1988).

Reversible differentiation occurs in certain retinoid sensitive

tumor cell lines. The effect of retinoids on melanogeneisis of murine or human melanoma cells is again dependent on the cell line and is reversible upon removal of the retinoid from the growth media. Retinoic acid induces an increase in tyrosinase activity and melanogenesis in S91 murine melanoma (Lotan and Lotan, 1980), murine melanoma B16F1 cells (Edward et al., 1988), and Hs939 human melanoma cells (Lotan and Lotan, 1981). Retinoic acid has been shown not to effect melanogenesis of B16F10 and B16BL6 murine melanoma cells, and to inhibit melanogenesis of UCT-Mel2 human melanoma cells (Hoal et al., 1982). Some tumor cell lines have been reported to undergo differentiation with addition of the retinoids. These include human neuroblastoma (LA-N-1) (Sidell, 1982), rat adenocarcinoma and keratinizing squamous cell carcinoma (Marchok et al., 1981).

Retinoids and Inhibition of Proliferation

The ability of retinoids to induce differentiation of tumor cells is confined to a few cell lines, while the inhibition of proliferation by retinoids is more commonly observed. Inhibition of proliferation in either tiisue culture or the soft agar assay is concentration dependent, time dependent, independent of cell density, and reversible. The natural retinoids with the most potent effects on tumor cell proliferation are 13 cis and all trans-retinoic acid (Lotan, 1980). The amount of RA induced inhibition varies from having no effect on proliferation (<10% inhibition) in some tumor cell lines to inhibiting proliferation by greater than 75 percent in other tumor cell lines (Lotan and Nicolson, 1977). Two of the tumor cell lines shown to be highly sensitive to retinoic acid were the murine melanoma S91 and B16

cell lines. Differences in sensitivity to retinoic acid were observed in clones of the B16 cell line. The parental B16 line as well as *in vivo* selected B16F1 and B16F10n (selected for brain metastasis) cells were extremely sensitive, while the *in vivo* selected B16F10, B16F100 (selected for ovarian metastasis), and the *in vitro* selected B16BL6 cells were less sensitive to retinoic acid (Lotan and Nicolson, 1979; Edward et al., 1988). An important clinical application for the assessment of retinoids in treatment of cancer was made using an *in vitro* tumor colony forming assay (Meyskens et al., 1983). This group had shown that a correlation existed between a clinical response and inhibition of colony formation of human melanoma cells by cytotoxic drugs in the soft agar assay (Meyskens et al., 1981). Four of the twenty-five human melanomas obtained from fresh biopsies were sensitive (reduction of 70% or more) to 13 cis-RA treatment and three of twenty were sensitive to 4 hydroxyphenyl-all-trans-retinamide (4-HPR) (a synthetic retinoid) treatment (Meyskens et al., 1983).

The inhibition of proliferation induced by retinoids is believed to be linked to a block in the progression of cells through the G_1 phase of the cell cycle (for review see Roberts and Sporn, 1984). Flow microflurometric analysis of S91 melanoma cells showed that retinoic acid increased the amount of cells in the G_1 phase and slowed the rate of entry of cells into the S phase of the cell cycle (Lotan et al., 1981). Niles (1987) has shown that inhibition of proliferation in G1 occurs without an inhibition of protein synthesis.

Retinoids and Changes in Morphology

Morphological changes and differences in the pattern of protein

synthesis have been noted in retinoic acid-sensitive tumor cell lines. Some phenotypic changes are seen in terminally differentiated F9 embryonal carcinoma cells (Linder et al., 1981; Lehtonen et al., 1983). Changes in morphology were characterized by progressive flattening and were shown to be time dependent taking approximately five days. Retinoic acid has been shown to induce the synthesis of type IV collagen, laminin, the intermediate protein vimentin, and the actin-myosin related protein tropomyosin (Linder et al., 1981). Retinoic acid treatment was shown to induce after two days the formation of vinculin rich adhesion plaques followed by the appearance of actin stress fibers (Lehtonen et al., 1983). The change in the distribution of vimentin was profound. Vimentin in untreated cells was found around the nucleus, while RA-treated cells had a distinct fibrillar pattern throughout the cytoplasm. Another intermediate protein, keratin, was found to be induced in many of the differentiating F9 cells (Lehtonen et al., 1983).

Morphological changes induced by retinoic acid have also been characterized in a rat osteogenic sarcoma cell line (UMR 106-06) (Ng et al., 1985). The phenotypic changes are apparently related to both cellular adherence and cell size. Retinoic acid-treated cells were larger and more adherant to the substrate. Changes in size and adherence were correlated by immunofluorescence and transmission electron microscopy to increases in microfilaments (stress fibers) and microtubules (Ng et al., 1985). Retinoic acid has also been observed to induce in the apparently undifferentiated mononuclear rhabdomyosarcoma cells the formation of thick and thin myofilaments

(Gabbert et al., 1988). Changes in the tumor phenotype induced by retinoic acid in other cell lines have been noted but are not well characterized. For example, retinoic acid induced progressive flattening in the morphology of three human osteosarcoma and two chondrosarcoma cell lines (Thein and Lotan, 1982). However, no characterization of the morphological changes were performed in these human sarcoma cell lines (Thein and Lotan, 1982).

Changes in the tumor cell surface have been linked to alterations in malignancy and metastasis (Stanford et al., 1986; Gabius et al., 1987). Retinoic acid induces changes in cell surface glycoproteins of a head and neck squamous cell carcinoma (1483) (Lotan et al., 1987a), cervical carcinoma (HeLa) (Lotan et al., 1980), and murine S91 melanoma cells (Lotan et al., 1983; 1987b). Retinoic acid treatment increases the radioactive labelling of high molecular weight (MW>400,000) galactoglycoconjugates and sialoglycoconjugates and a 280,000 MW sialoglycoconjugate in a well differentiated head and neck squamous cell carcinoma cell line. A less well differentiated head and neck tumor was not sensitive to retinoic acid (Lotan et al., 1987a). The varied effect of retinoic acid on the the head and neck carcinoma cell lines was believed to be related to the state of differentiation of the tumor, the control of tumor cell proliferation and surface markers. A correlation apparently also exists between homotypic aggregation and metastasis (Updyke and Nicolson, 1987). Retinoic acid has been shown to decrease homotypic aggregation of S91 melanoma cells (Lotan et al., 1987). A link between the induction by retinoic acid of glycosylation of a 160,000 MW sialoglycoconjugate and decreased homotypic aggregation

may be important (Lotan et al., 1987).

Inhibition of proliferation by retinoic acid is not necessary in some cases to induce phenotypic changes in tumor cells. All trans-retinoic acid has been shown to reduce production and activity of type IV collagenase in rat mammary adenocarcinoma (13762NF) cells at a concentration that did not inhibit proliferation (Nakajima e tal., 1989). Growth inhibition was also not observed with 13 cis retinoic acid in murine squamous carcinoma (KLN205) cells. However, 13 cis-retinoic acid caused an increase in expression of lectin receptors (Couch et al., 1987). The alterations in lectin receptors might have a role in the induced decrease seen in experimental metastasis by RA-pretreated murine squamous carcinoma (KLN205) cells by increasing natural killer cell activity (Couch et al., 1988).

Retinoids and In Vivo Control of Cancer

The evidence is strong that retinoids can inhibit proliferation, induce differentiation, and change the morphology of many tumor cell lines *in vitro*. Evidence is also accumulating for a role of retinoids in the therapeutic control of human tumors and metastasis (for a review see Lippman et al., 1985). Retinoids have a role in decreasing tumor size and tumorgenicity in animal models (Felix et al., 1975; Drewa and Schachtschabel, 1985; Olsson et al., 1985; Halter et al., 1988; McCue et al., 1988; Dillehay et al., 1989). There is a link between a decrease in proliferation *in vitro* and decrease in tumorgencity *in vivo*. For example, all human squamous-cell (RH-SCL-L11) and small-cell (RH-SCC-L10) lung carcinoma clones found to be sensitive to RA in the soft agar assay were found not to be tumorgenic in nude mice after *in*

vitro pretreatment (Olsson et al., 1985). 13 cis-retinoic acid has been shown enhance T cell protection. Dillehay et al. (1989) treated preimmunized mice, athymic mice and euthymic mice with 13 cis-RA. Only the euthymic mice had decreased lymphoma tumor growth (Dillehay et al., 1989).

The results of pretreatment of tumor cells in vitro with RA before tail vein inoculation (experimental metastasis) are not as simple. In some investigations, RA pretreatment in vitro caused a decrease in lung colonies (Couch et al., 1988 and Edward et al., 1989), while pretreatment of F9 embryonal carcinoma cells with RA and dibutyl cAMP caused a change in the pattern of organ metastasis (Terrana et al., 1987). However, the change in pattern of organ metastasis might have been induced by the dibutyl cAMP.

A role for retinoids (without pretreatment) in the reduction of metastasis of a hamster melanoma cell line (HM1-F5) in athymic mice has been established by Schleicher et al. (1988). The number of spontaneous metastases was shown to be decreased by 13 cis-RA and two synthetic retinoids (2 hydroethyl retinamide and N-4(-hydroxyl-phenyl) all trans retinamide (4HPR)). One of the synthetic retinoids (4HPR) was also shown to decrease the incidence of metastasis (Schleicher et al., 1988).

Retinoids and Clinical Control of Cancer

Retinoids have been used in the clinic for the treatment of neoplastic disease (for review see Lippman et al., 1987). Retinoids appear to have value in the treatment of basal cell carcinoma, advanced regional or metastatic cutaneous squamous cell carcinoma, melanoma,

mycosis fungoides (an uncommon T-cell lymphoproliferative disease), leukemia, and squamous cell cancer of the head and neck. Topical all trans-RA (tretinoin) was reported to produce 16 complete responses and 32 partial responses in 49 patients with basal cell carcinoma (Schumacher and Stuttgen, 1971; Belisario, 1972; Bollag and Ott, 1975; Sankowski et al., 1984). Oral 13 cis-RA was reported to produce a complete response in 39 lesions and a partial response in 162 lesions (out of 248 lesions in 11 patients having multiple lesion basal carcinoma) (Peck et al., 1979). Oral 13 cis-RA produced 1 partial response in a patient with basal cell carcinoma (Meyskens et al., 1982). A synthetic retinoid, oral etretinate, was effective in treating basal carcinoma with 3 complete responses 14 partial responses and 14 minor responses reported in 40 patients. The reports on retinoid treatment in advanced cutaneous squamous cell carcinoma are limited to four studies with a total of 14 patients evaluated. Oral etretinate produced one complete response and one partial response (out of 4 patients) (Grupper and Berretti, 1983). Another synthetic oral retinoid, arotinoid, was shown to produce one partial response (out of one patient evaluated) (Kingston et al., 1983). Oral 13 cis-retinoic acid produced 2 complete responses and 5 partial responses (out of nine patients evaluated) (Meyskens et al., 1982; Lippman and Meyskens, 1987). Successful treatment of mycosis fungoides has been reported with systemic 13 cis-retinoic acid (Warrell et al., 1983; Molin et al., 1985; Kessler et al., 1987) and systemic etretinate (Souteyrand et al., 1981; Zachariae et al., 1981; Claudy and Rouchouse, 1985). The clinical data is limited with leukemias, but there is promise for

retinoic acid in the treatment of acute promyelocytic leukemia (Flynn et al., 1983). Systemic 13-cis RA showed some efficacy in the treatment of advanced head and neck cancer with 3 partial responses observed out of 19 patients (Meyskens et al., 1982).

Treatment of patients with topical all trans-retinoic acid or oral 13 cis-retinoic acid with either cutaneous and metastatic melanoma have met with success, but have been limited in scope. Topical all trans-retinoic acid used to treat two patients with cutaneous melanoma produced a complete regression in one patient and a partial regression in the other (Levine and Meyskens, 1980). Partial responses were observed in three out of 20 patients that received oral 13 cis-retinoic acid in a phase II trial (Meyskens et al., 1982).

Acute and Chronic Toxicity of Retinoids

In vivo animal experiments and clinical trials with retinoids have been limited by both acute and chronic toxicity (for a review see Kamm et al., 1984). Dosage and timings are critical in evaluating potential antitumor effects. Major side effects of acute toxicity include central nervous system symptoms such as severe headaches, increased cerbrospinal fluid pressure, irritablity and dizziness, nausea, vomiting, fatigue, weakness, and somnolence. Minor side effects of acute toxicity include skin disorders such as inflammation of the lips, dryness and swelling of the skin, and dry mucous membranes. Eye disorders are also common. They include conjunctival inflammation, dryness, diplopia, and blurry vision. Tenderness of the long bones, an enlarged liver or spleen and edema are also commonly seen in acute toxicity.

Major effects of chronic toxicity include abnormalities in reproductive function and embryological development, defects in liver function and the skeleton. Hypertriglyceridermia is also observed. Minor chronic side effects include dry skin and mucous membranes, itching inflammation of the lips, hair loss, visual disturbances and occipital headaches (Koerner and Voellm, 1975). Synthetic retinoids may be critical for possible therapeutic trials. Synthetic retinoids tend to have less severe and fewer central nervous system and liver side effects (Lippman et al., 1987). Arotinoids, for example, can be used at lower concentrations and still have effects in vitro simliar to retinoic acid (Klaus et al., 1983).

Butyric Acid

I have investigated another differentiation agent, butyric acid. Butyric acid (and its sodium salt) is a natural four carbon fatty acid normally present in lipids and is formed by the hydrolysis of ethylbutyrate. Much less research has been done on the role of butyric acid in the normal functioning of an organism. Most of what is known is based on in vitro studies on normal and tumor cell lines. As with other differentiation agents, butyric acid has been shown to induce differentiation, inhibit proliferation, induce changes in morphology, and reduce tumorgenicity of various tumor cell lines (for a review see, Kruh, 1982). Nontoxic BA concentrations are at the millimolar level (less than 5 mM), though the cytostatic and noncytotoxic concentrations vary from cell line to cell line. Thus butyrate levels needed to induce functional changes in cells are much more than the concentrations of various retinoids (from 10^{-6} to 10^{-9} M).

Butyrate and Differentiation

Butyate induces reversible differentiation in many tumor cell lines. The first report of differentiation induced by butyrate was in murine Friend virus-transformed erythroleukemia cells that had been shown to synthesize hemoglobin (Leder et al., 1975). The embryonal carcinoma cell lines, as observed with the experiments with retinoic acid, presents an interesting model for differentiation. The PCC4(RA)$^{-}$1 cell line that is resistant to differentiation by retinoic acid has been shown to be partially differentiated by BA. However, further differentiation takes place when retinoic acid and BA are added together (McCue et al., 1984). In contrast, BA has been shown to inhibit the differentiation of RA-treated F9 embryonal carcinoma cells (Levine et al., 1984), and stimulate the differentiation of 6050AJ and PCC4azaR embryonal carcinoma cells (McCue et al., 1984). Thus, these similiar cell lines may be used in studies to elucidate the action of RA and BA at the molecular level.

As with other differntiating agents, the effect of BA on melanoma cells is unique. Tryrosinase production (a classical marker for differentiation) is inhibited by BA in murine B16F10 melanoma cells (Malik et al., 1987). However, BA stimulated the activity of other enzymes including NADPH cytochrome c reductase (indictive of the development of the endoplasmic reticulum) (Nordenberg et al., 1987). In a human melanoma (SKMEL-28) cell line, BA has been shown to induce marker enzymes such as alkaline phosphatase, y-glutamyl transpeptidase, and NADPH cytochrome c reductase(Nordenberg et al., 1987). Thus, BA increases protein production in ways not previously observed in cells

induced to differentiate by other agents.

Butyrate and Inhibition of Proliferation

Butyrate induced reversible inhibition of proliferation is sometimes accompanied by induction of marker enzymes that are indicative of differentiation. Human tumor cell lines that have been shown to be sensitive to butyrate induced inhibition of proliferation include two colonic carcinomas (SW 480 and Sw 620) (Kim et al., 1980), three pancreatic adenocarcinomas (MIA PaCa, PANC-1, and CAPAN-1) (McIntyre and Kim, 1984), two poorly differentiated ovarian adenocarcinomas (PE01 and PE04)(Langdon et al., 1988); one salivary adenocarcinoma (HSG) (Azuma et al., 1986), one squamous cell carcinoma of the tongue (HN1) (Bijman et al., 1987), and a human melanoma (SKMEL-28) (Nordenberg et al., 1987). Inhibition of soft agar clonogenicity was also induced by BA (Nordenberg et al., 1987). The soft agar assay is believed to be a good indicator for tumorgenicity (Meyskens et al., 1983). Arrest of proliferation has been shown to occur in the G1 phase of the cell cycle (RA arrests proliferation in G1) (Thorgeirsson et al., 1984).

Butyric acid inhibits proliferation in cell lines that are not sensitive to RA. Retinoic acid did not inhibit proliferation but did induce alkaline phosphatase activity in 9-1C rat prostatic adenocarcinoma cells. Butyric acid, on the other hand, inhibited proliferation at a concentration of 5 mM or more (Reese et al., 1985). Butyrate was also the most effective short chain fatty acid in the induction of alkaline phosphastase activity, inhibition of proliferation, and increasing the protein content of 9-1C cells (Reese

et al., 1985).

Butyrate and Changes in Morphology

Changes in proliferation induced by butyrate are usually accompanied by dramatic time dependent changes in morphology. The most characterized changes in morphology are in virus transformed fibroblasts (KNRK) (Altenburg et al., 1976; Ryan and Higgins, 1988) and rat hepatoma (72/22) cells (Borenfreund et al., 1980; Ryan et al., 1987). In virus transformed fibroblasts, butyrate was shown to produce the formation of long cytoplasmic processes within 12 to 24 hrs after which there was a progressive flattening of cells at 72 hr. Ultrastructural and immunofluorescent analysis of the KNRK cells found that there was a dramatic increase in the amount of microfilaments and microtubules, and an enhanced formation of adhesion plaques (Altenburg et al., 1976). The changes in KNRK cells were further characterized by gel electrophoresis (Ryan and Higgins, 1987). There was a 2.5 to 3.0 fold increase in actin, alpha actinin (an actin binding protein), and fibronectin. However, the amount of tropomyosin, vimentin, alpha and beta tubulin in the KNRK cells was found to be significantly reduced (Ryan and Higgins, 1987). The reduction in the amount of alpha and beta tubulin conflicts with the results in the experiments of Altenberg et al. (1976). The electrophoresis pattern of butyrate-treated KNRK cells appear to contain little if any alpha or beta tubulin. This is odd noting the elaboration of microtubules in KNRK cells (Altenburg et al., 1976).

Reorganization of the cytoskeleton with inhibition of proliferation also occurs in 72/22 cells treated with butyrate

(Borenfreund et al., 1980; Ryan et al., 1987). F-actin was observed to accumulate at the cellular edges (peripheral band) and in the central portion of the cell. The total actin was measured by densitometry of electrophoretic patterns and flow cytometry. Total actin was found to be increased in butyrate-treated 72/22 cells without an increase in mRNA. The structure of the intermediate filaments, which consists of both vimentin and keratin, in 72/22 cells, was also changed by treatment with butyrate (Borenfreund et al., 1980). Untreated hepatoma cells have been shown to have distinct genetically stable, acentric, abnormal, juxtanuclear aggregates composed of intermediate filaments (Borenfreund et al., 1978). Butyrate treatment induces a progressive disintegration of the aggregrates with a resultant normal display of filaments (Borenfreund et al., 1980). A flattened morphology is typical of BA-treated cells including B16F10 cells (Nordenberg et al., 1986), but a further characterization of cytoskeletal changes is needed. Most morphological changes produced by butyrate in many tumor cell lines are reversible within three days. However, changes persisted after 14 months without butyrate in the human salivary adenocarcinoma cells (Azuma et al., 1986).

Butyrate and In Vivo Control of Cancer

Few reports have investigated the role of butyrate in the in vivo or clinical settings. A correlation was shown to exist in the delay of tumor appearance, decreased size of tumors and BA-pretreatment of B16F10 cells (Nordenberg et al., 1986). However, a correlation for a decrease in tumorgenicity and BA-treatment was not observed in human colon cancer cells (Kim et al., 1980). BA-pretreatment has been

observed to produce, as with other differentiation agents such as DMSO, a concentration and time dependent increase in experimental metastasis of low metastatic Lewis lung carcinoma (P-29) cells (Takenaga, 1986). Only two studies have reported using butyrate in a therapeutic setting (Bourgeade et al., 1979; Novogrodsky et al., 1983). Butyrate and interferon were administered (i.p.) on alternate days to Swiss or DBA/2 mice that received murine sarcoma (180 TG) cells (i.p.). Treatment with BA and interferon was found to increase mean survival time and final survival rate over untreated or interferon treated mice (Bourgeade et al., 1979).

The effect of butyrate on cells from peripheral blood of five human patients with acute leukemia was also examined in vitro (Novogrodosky et al., 1983). One patient (a five year old boy) that had acute myelogenous leukemia received 2% butyrate (500 ml/kg/24 hrs) for ten days. After this time period, it was observed that myeloblasts were eliminated from the peripheral blood, the number of mature myeloid cells was increased, and the number of bone marrow myeloblasts was reduced by 70 to 80%. There were no signs of toxicity in the animal study or clinical trial.

The Synergistic Action of Differentiation Agents

The use of differentiation agents as adjuvant or in combination with cytotoxic drugs or other differentiation agents may be an important concept for preventing tumor progression. The notion has met with some success in vitro. Butyrate, in combination with retinoic acid, produced an inhibition of proliferation of human retinoblastoma (Y-79) that was synergistic in nature (Kyritis et al., 1984). The

addition of PGA_1 to media containing both dexamethasone (a synthetic glucocorticoid) and all trans-retinoic acid was shown to synergistically inhibit the colony forming ability of C8146c human melanoma cells (Bregman et al., 1983). A similiar enhanced inhibition of colony forming ability was found with the combination of dexamethasone, alpha interferon, all trans retinoic acid, and DFMO (Bregman and Meyskens, 1986). This in vitro study by Bregman and Meyskens (1986) is important since the drug concentrations used in vitro were within the limits that are pharmalogically achievable in the clinical setting.

The Seed and Soil Theory in Metastasis

Much of the experimental ground work of the current theories of tumor cell metastasis comes from experiments done on the B16 cell line. Fidler's 1970 study found that only 0.1% of tumor emboli survive after 14 days to give rise to lung nodules after tail vein injection (experimental metastasis assay). A great deal of information has been gleened since this time using the B16 cell line that substantiates much of the 'seed and soil' 1889 theory of Paget. Paget proposed that metastasis was the result of certain tumor cells ("seeds") to find an appropriate organ microenvironment ("soil") to develop and grow. Paget believed this process was nonrandom and only partially due to circulation patterns and mechanical lodgement. Clinical examples of metastasis believed not to be due to random events include ocular and cutaneous malignant melanoma. The primary sites for these melanomas are in the eye and skin, respectively. Ocular melanoma metastasizes primarily to the liver, while cutaneous melanoma metastasizes primarily

to the brain, liver and bowel. The parent B16 melanoma (primary) tumor was found to be heterogeneous in regard to metastasis (Poste et al., 1981). An indication of this was the ability to select for the highly metastatic cells by in vivo (Fidler, 1973; Fidler and Nicolson, 1976) or in vitro methods (Hart, 1979). Metastasis resulted from prexisting variant cells within the parental tumor rather than an adaptation to a specific site environment (Brunson and Nicolson, 1980; Nicolson and Custead, 1982). This is the seed part of the hypothesis. The 'soil' experiments included studies that utilized ectopic grafts. B16F10 cells metastazied not only to the lung but to subcutaneous lung tissue grafts (Hart and Fidler, 1980).

The Cytoskeleton and Malignancy

There are three major classes of cytoskeletal filaments found in eukaryotic cells: microfilaments, microtubules, and intermediate filaments. The cytoskeleton has many different functions essential for the normal functioning of the cell including such diverse activities as intracellular transport, mitosis, control of cellular proliferation, ciliary movement, cellular migration, and maintenance of cell shape. Microfilaments are 5-7 nm in thickness and composed of F-actin (filamentous actin) which is polymerized from globular monomers (G-actin). F-actin and G-actin interact with numerous actin associated proteins that regulate the length, distribution, and stability of microfilaments. An important probe for analysis of actin filaments is the cyclic oligopeptide phallodin. Phalloidin, which is derived from a toadstool (Amanita phalloides), binds to actin filaments stoichiometrically (Miyamoto et al., 1986). Microtubules are

approximately 25 nm in diameter. The wall of the microtubule is composed of protofilaments consisting of two polypeptides, alpha and beta tubulin. Attached to the walls of the microtubules are numerous microtubule associated proteins.

In the discussion of the changes induced in tumor cells by various differentiation agents, one of most obvious are phenotypic changes. Investigators have used immunofluorescence since 1975 to speculate on the role of the cytoskeleton in neoplastic transformation (Brinkley et al., 1975; Puck, 1977). Current thought attributes the decrease in microtubules observed in transformed cells by Brinkley et al. (1975) and Puck (1977) to the shape and spreading of transformed cells that obscured the microtubule networks in immunofluorescent studies (DeMey et al., 1978; Tucker et al., 1978; Asch et al., 1979). Laboratories are now using immunofluorescence and electron microscopy to find changes in tumor cell cytoskeleton and are correlating possible alterations with biochemical analysis. Immunofluorescence is not easily quantifiable given the variable nature of the interactions between antibody and cytoskeleton antigen or antigens.

Malignant transformation has been found to induce many different cytoskeletal changes in cell lines. Whether cytoskeletal changes are found only in specific cell lines needs further study. Kirsten virus-transformed rat NRK cells were found to have almost twice as much pelletable tubulin compared to NRK cells using a colchicine binding assay and a stabilization buffer (Rubin and Warren, 1978). The amount of tubulin (<u>without</u> the use of a stabilization buffer) was found by incorporation of ^{35}S methionine not to be decreased in SV-40

transformed murine Swiss 3T3 or Rous sarcoma virus-transformed NRK cells, but there was a two fold decrease in calmodulin (Chafouleas et al., 1981). Calmodulin is a protein present in most eukaryotic cells that binds calcium with high affinity and specificity (for a review see Means and Dedman, 1980). Calmodulin has a number of functions including Ca^{+2}-dependent regulation of microtubule assembly-diassembly (Schiwa et al., 1981), cell cycle regulation (Chafoules et al., 1982), and conferring stablity to DNA in tumor cells (Lonn and Lonn, 1986). Calmodulin has been shown by immunofluorescence to be closely associated with the cytoplasmic microtubule complex (Deery, 1986). Beta-tubulin has been shown to be composed of different isotypes (for review see Sullivan, 1988). A unique tubulin isotype (hB_4) was found by immunofluorescence to be expressed in high levels in tumors of epithelial origin as compared to normal tissues. The expression of this isotype was not as distinctive with cultured malignant cell lines (Walker et al., 1987). This is one example of the concerns in using cultured cell lines.

Microfilaments and Malignancy

Morphological changes in neoplastic transformation have been studied with respect to alterations in microfilaments (Pollack et al., 1975; Rubin et al., 1978; Tucker et al., 1978). Immunofluroescence and phallocidin flourescent studies have noted in some tumor cell lines (including the B16 cell line) the lack of stress fibers and accumulation of structures called F-actin aggregates (Carely et al., 1981). Aggregates contain not only F-actin but also alpha actinin, vinculin, talin and fimbrin but not tropomyosin (Carely et al., 1986).

Vinculin, talin and fimbrin are proteins found in adhesion plaques (David-Pfeuty and Singer, 1980; Burridge and Connell, 1983; Carely et al., 1986). Adhesion plaques and substrate interactions are apparently weakened or disrupted in neoplastic transformation (Nigg et al., 1986). Vinculin is a target for tyrosine phosphorylation by the src oncogene (Sefton et al., 1981). Reorganization of vinculin, actin, and alpha-actinin into aggregates was observed in virally transformed cells that were encoded for the production of tyrosine kinases. In contrast, cells transformed by viruses without tyrosine kinase activity did not produce aggregates (Nigg et al., 1986). Rous sarcoma virally transformed fibroblasts had less vinculin and talin than normal chick fibroblasts (Otto, 1985). Other modifications in adhesion plaques may occur in malignant transformation. Rous virus transformed chick fibroblasts also showed more than a three fold reduction in the acylation of vinculin (Burn and Burger, 1985). Alpha-actinin is associated with adhesion plaques and stress fibers (Mangeat and Burridge, 1984). Stress fibers are large bundles of microfilaments, thought to be a higher level of organization of actin filaments. These results are important since, it has been observed in a number of transformed fibroblast cell lines, that there was a decrease in the levels of one or two major tropomyosins and an increase in the levels of one or two minor tropomyosins (Matsumura et al., 1983). These experiments were done by biochemical analysis of isolated microfilaments (Matsumura et al., 1983). Alpha-actinin and tropomyosin isotypes have an apparent function in stabilization of stress fibers. Alpha-actinin was colocalized with actin to the leading edge of motile

rabbit V2 carcinoma cells, while tropomyosin was not observed in the motile leading edge (Jockusch et al., 1983). It is interesting to note an increase in a-actinin was observed in butyrate-treated KNRK cells with a decrease in tropomyosin (Ryan and Higgins, 1987). An increase in migration induced by BA-treatment may be a consequence of an increase in alpha-actinin (McGarvey and Persky, 1989).

The Cytoskeleton and Metastasis

Many of the various stages involved in tumor cell metastasis are related to the cell's ability to deform, to make and break intracellular contacts, and to actively migrate. These processes are known to be controlled by the components of the cytoskeleton (Raz and Geiger, 1982). The evaluation of possible differences between the cytoskeletons of low and high metastatic cells has been limited by the same problems in experiments looking for differences between transformed and normal cell cytoskeletons. These problems include the the use of immunofluorescence, which is at best only semiquantitative, and the use of a limited number of tumor cell lines. Whether changes seen in tissue culture are valid in vivo is another question. The use of biochemical techniques may be helpful to find if there are any actual differences between high and low metastatic cells.

Immunofluorescent studies have shown that highly metastatic variants of the murine K-1735 melanoma and of the UV2237 fibrosarcoma have been shown to exhibit distorted actin bundles, a reduction in the number of focal contacts, and a reduced organization of vinculin (Raz and Geiger, 1982). These results were confirmed using various clones of the K-1735 cell line (Volk et al., 1984), in the Dunning R3327 rat

prostatic adenocarcinoma system (Zachary et al., 1986), and rat pancreatic adenocarcinoma (BSp73) variants (Raz et al., 1986). The loss of actin organization was shown to be an important part of the transition from noninvasive benign human colonic tumors to invasive malignant tumors (Friedman et al., 1984). On the other hand, three distinct phenotypes were observed in human breast carcinoma cells and the cytoskeleton seemed to be more dependent on cell shape (Brinkley et al., 1980). In addition, the organization of the cytoskeleton in the rat adenocarcinoma cells varied between clones, not with the metastatic potential of the clones (Lichtner and Nicolson, 1987).

Biochemical studies may further define the role of the cytoskeleton in metastasis. The cytoskeletal core actin (Triton X insoluble) assembly was increased by 35% in highly metastatic clones of B16F10 cells grown in the presence of fetal calf serum. This increase was measured by the DNase I inhbition assay and was not seen in the low metastatic clones (Holme et al., 1987). Parental and low metastatic B16 cells also displayed a newly found actin polypeptide (A^X), which comprised 30% of the total actin. This peptide, isolated by a number of biochemical techniques including the microfilament isolation techique of Matsumura et al. (1983) and two dimensional electrophoresis, was not found to be expressed in highly metastatic B16BL6 or B16F10 cells (Taniguchi et al., 1986). Given the importance of actin in motility and cell shape, one can only speculate on the role of different actin isotypes or the assembly patterns of actin in metastatis.

The synthesis and expression of intermediate filament types is

specific for tissue types (Franke et al., 1978). Melanoma cells synthesize intermediate filaments composed only of vimentin (Ramaekers et al., 1983). The synthesis of vimentin has been shown to be decreased in the rounded configuration, highly metastatic variant of the B16-F10 cell line (Raz and Ben-Ze'ev, 1983). Modifications of cell shape are apparently important in metastasis (Stackpole et al., 1985), but changes in vimentin synthesis may be only part of the story (Nabi and Raz, 1987). On the other hand, cyclohexamide decreased the synthesis of vimentin (as well as actin and tubulin) and also decreased lung nodule formation (Ben-Ze'ev and Raz, 1985). Is a decrease in vimentin associated with a decrease or increase in metastasis? The effect of cyclohexamide on metastasis might not be directly due to the disorganization or decrease in the synthesis of vimentin, but due to a decrease in general protein synthesis (Thorgeirsson et al., 1984).

Further characterization of the role of the cytoskeleton in metastatic spread is needed. Agents that have disruptive effects on the cytoskeleton are important tools, but given the wide range of functions that the cytoskeleton has in a cell, dissecting a specific role for the cytoskeleton in individual steps of the metastatic cascade will be a difficult job. The microfilament system appears to have a crucial role in lung implantation. B16F1 and B16F10 cells pretreated with tertiary amine local anesthetics have decreased lung colonization abilties (Nicolson et al., 1986). Tertiary amine local anesthetics result in cell rounding and the loss of stress fibers (Nicolson et al., 1976). However, pretreatment of B16 cells with either cytochalasin B (an actin depolymerization agent) and colchicine (an antimicrotubule

agent) decreased lung colonization with a surprising increase in extrapulmonary metastasis (Hart et al., 1980). These three agents have been shown to decrease homotypic adhesion and endothelial cell adhesion (Hart et al., 1980; Nicolson et al., 1986). Further work is also required to find the mechanisms in which cytochalasin B and colchicine increased extrapulmonary metastasis.

Metastasis is a complex series of events that can be investigated by holistic and reductionistic experiments. We attempt using in vitro models to understand the overall process of metastasis under specific, defined conditions that reduce the numbers of variables. The revelance of each in vitro model will become known only when in vivo metastasis is understood.

Chapter III

PURPOSE

This dissertation will examine the affect of two antitumor drugs, all trans-retinoic acid (RA) and butyric acid (BA), on B16a murine melanoma cells. The five parameters to be investigated are: (1) cytotstatic and cytotoxic concentrations, (2) in vitro migration, (3) in vitro invasion, (4) in vivo experimental metastasis (tail vein injection), and (5) cytoskeletal morphology. Retinoic acid and BA can be less host toxic and less mutagenic than conventional chemotherapy when administered at cytostatic concentrations. Both drugs deserve investigation because their effect on the individual steps of the metastatic cascade is undefined. My hypothesis, which is supported by my in vitro preliminary studies and by the literature, is that BA inhibits growth yet increases in vitro migration, in vitro invasion, and in vivo experimental metastasis. In contrast, RA suppresses migration, invasion, and experimental metastasis. My study stresses the importance of testing the invasive and metastatic effects of potential antitumor agents. The net effect of RA and BA on invasion and metastasis may be due to their differential action on the cytoskeleton. This project may lead to a further understanding of the metastatic process. In Chapter IV, I determined the effect of RA and BA on the proliferation, migration and cytoskeleton. In Chapter V, I determined the effect of RA and BA on adhesion, invasion, and in vivo experimental metastasis. In Chapter IV, I quantitated the effect of migration, invasion, RA and BA on the actin cytoskeleton.

CHAPTER IV

THE EFFECTS OF RETINOIC ACID AND BUTYRIC ACID ON IN VITRO MIGRATION BY MURINE B16a MELANOMA CELLS: A QUANTITATIVE SCANNING ELECTRON MICROSCOPIC STUDY

ABSTRACT

Retinoic acid (RA) and butyric acid (BA) were investigated for their effect on in vitro migration of highly metastatic murine B16a melanoma cells. These potential antitumor agents are known to alter the cytoskeleton. Our initial studies determined the 72 hr cytostatic/cytotoxic concentration of RA (1×10^{-6} M/>1×10^{-5} M) and BA (1.5 mM/2 mM). Cytostasis by RA and BA was confirmed by autoradiography and radioisotope incorporation. For the migration assays, cells were plated on 3 um and 5 um diameter pore polycarbonate membranes. Complete media was added containing RA or BA at the time of plating. For BA-pretreatment studies, BA was added to cells for 72 hr prior to plating cells on the membranes in fresh media + BA. Top and bottom surfaces were examined after 72 hr of incubation by scanning electron microscopy. Although RA and BA induced cells on the top of the membrane to change morphology as shown by phase contrast, transmission, and scanning electron microscopy, only BA enhanced the migration of cells to allow for passage through the 3 um diameter pores. Butyric acid enhanced migration through the 3 um diameter pore membranes by 511%. For the 5 um pore membranes, 55% of the plated number of untreated early passage cells migrated to the bottom surface as compared to 57% for the BA-treated cells and 15% for RA-treated cells. However, if cellular proliferation over the 72 hr period was factored in, BA-treatment increased migration by 456% and pretreatment of cells with BA increased migration by 893%. Without considering proliferation, RA inhibited migration by 75% over untreated cells. The

decrease in migration observed in RA-treated cells was due to an inhibitory effect on cellular migration and decrease in proliferation.

INTRODUCTION

Progress in the treatment of cancer has been hampered by the inherant ability of tumor cells to metastasize. Metastasis is a complex, multistep process that is poorly understood. Tumor cells must invade the extracellular matrix (ECM) at multiple stages during the metastatic process. Tumor cell invasion of the ECM has been proposed to be a three step process: (1) attachment to endothelial cells and the ECM, (2) proteolytic digestion of the ECM, and (3) locomotion (Liotta et al., 1983). Although the role of tumor cell locomotion in invasion and metastasis has been studied (Werling et al., 1986; Raz and Ben-Ze'ev, 1987; Maslow, 1987; Verschueren et al., 1988), the specific step of migration needs to be clearly distinguished from the overall process of metastasis (Grimstad, 1987, 1988).

The modified Boyden chamber has been a major instrument to quantitate migration (Varani et al., 1978; Nabeshima et al., 1986; Fliegel et al., 1985; 1986; Wewer et al., 1987). Membrane invasion culture system (MICS) chambers, (Gehlsen et al., 1984), Transwell chambers (Repesh, 1989) and numerous other chambers that are modifications of the Boyden chamber have been used to evaluate migration and invasion. Early studies on quantitating migration in the Boyden chamber (Varani et al., 1978) have now evolved to analyze the effect on migration by various chemotactic agents (Orr et al., 1978; Lam et al., 1981; Thorgeirsson et al., 1982; Varani et al., 1985), haptotaxic agents (McCarthy and Furcht, 1984; McCarthy et al., 1986) and drugs (Spiro and Mundy, 1980; Daughaday et al., 1981). Light

microscopy (Thorgeirsson et al., 1982), radiolabelling (Repesh, 1989) and scanning electron microscopy (Albrecht-Buehler, 1986) have been used to evaluate cell locomotion and migration.

Agents that interfere with either microtubules (e.g. colchicine or taxol) or microfilaments (e.g. cytochalasin B) inhibit tumor cell migration (Spiro and Mundy, 1980). Raz and Geiger (1982) speculated that the role of the cytoskeleton was not only active in migration, but also in the tumor cell's ability to deform and make/break intercellular contact. Retinoic acid (RA) and butyric acid (BA) inhibit growth and induce differentiation of tumor cells (Nordenberg et al., 1986; Roberts and Sporn, 1984) as well as affect the cytoskeleton (Lehtonen et al., 1983; Ng et al., 1985; Borenfreund et al., 1980).

In my investigation, I quantitated by scanning electron microscopy (SEM) the migration of untreated, RA-treated, and BA-treated B16a cells through porous polycarbonate membranes. I calculated migration rates by SEM. The migration rate of B16a cells was defined as the number of cells adhering to the bottom of a polycarbonate membrane divided by the number of cells plated. The number of cells adhering to the bottom of the entire membrane was extrapolated from the number of cells counted within 50 random fields as observed by SEM. This study demonstrated that RA inhibited while BA increased the migration rate of B16a cells. I have also shown by transmission electron microscopy (TEM) that RA and BA induce changes in the cytoskeleton of B16a cells. The role of the cytoskeleton in tumor cell migration is discussed.

MATERIALS AND METHODS

Cell Culture

Murine B16a (amelanotic) melanoma cells were obtained from the DCT Tumor Repository (NCI Frederick Cancer Research Facility, Frederick, Maryland). Cells were cultured in Eagle's Minimum Essential Medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and l-glutamine supplemented with sodium pyruvate (110 mg/l) (Sigma, St Louis, MO), 10% heat inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin G-streptomycin sulfate-amphotericin B (Fungizone) (Gibco), MEM non-essential amino acids (10 ml/l) (Gibco), sodium bicarbonate (1.27 g/l) (Sigma), and Hepes (5.96 g/l) (Sigma). The final pH of the medium was 7.3. Cells were cultured in a humidified incubator (37^{o}C in 5% CO_2) and were refed every three days. Cells were subcultured with trypsin/EDTA (Sigma) in Hanks Balanced Salt Solution without Ca^{++} and Mg^{++} (Gibco) during the log phase of growth. All other parameters were standard. The experimental metastatic assay (tail vein injection) of Poste et al. (1980) was used to verify the lung-colonizing potential of the cells in C57BL6 mice. The metastatic potential of the cells was confirmed prior to initiating the migration assays and also after completing all assays.

In Vitro Growth Curves of RA, BA, Calf Serum, and Colchicine-Treated Cells

Cells were plated in triplicate for each experiment at a concentration of 150,000 cells per 35 mm dish (50,000 cells per ml). 24 h later, RA (0M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M in complete medium

containing 0.1% ethanol + 10% FBS), BA + 10% FBS (0.0mM, 0.1mM, 0.2mM, 0.5mM, 1mM, 1.5mM and 2mM in phosphate buffered saline), 10% calf serum (CS), and colchicine (0M, 10^{-8}M, 10^{-7}M and 10^{-5}M in complete medium containing 0.1% ethanol + 10% FBS) were added independently. Retinoic acid and colchicine were obtained from Sigma, BA from J.T. Baker (Phillipsburg, N.J.) and CS from Gibco. Different concentrations of RA were prepared under minimal light conditions according to the method of Lotan and Nicolson (1979). Retinoic acid was dissolved in 100% ethanol to produce a series of stock solutions from 10^{-1} to 10^{-5} M, and stored for up to a week at -60 ^{o}C. Stock solutions were diluted 1:1000 with complete medium prior to usage. Two sets of controls were run. One control contained complete medium. The second control contained 0.1% ethanol (the final ethanol concentration) in complete medium. The FCS was exposed to ultraviolet light for 1 h in order to destroy vitamin A activity (Ng et al., 1985). The concentration of RA in the control medium was therefore considered to be 0 M. The 10^{-5}M concentration of taxol used in the migration assay was the same concentration used in the motility assay of Keller and Zimmermann (1986). Viability and cell number were determined at 72 h by the trypan blue dye exclusion test and the hemacytometer, respectively. The data was reported as the mean number of cells $\pm$ standard deviation.

Incorporation of ^{3}H Thymidine by BA-treated Cells

The incorporation of ^{3}H-thymidine was determined by autoradiography and beta scintillation counting according to the method of Ryan et al. (1987). For autoradiography, untreated and BA-treated (1.5 mM) were incubated for 72 h on acid-washed glass slides. One

uCi/ml of ^{3}H-thymidine (specific activity 6.7 Ci/mM) was added 24 and 54 h after the start of incubation. Slides were washed with cold 0.1 M thymidine in phosphate buffered saline (PBS) and fixed for 2 min at room temperature in freshly prepared 4% paraformaldehyde in PBS (pH=7.4). Slides with unlabelled cells were run as a control. Slides were washed with PBS, dehydrated in ethanol and air dried. They were coated with Kodak NTB2 emulsion, dried overnight in the dark, placed in a light tight box and refrigerated for 2, 4, and 6 days. Slides were developed in D19 developer for 5 min. Each slide was rinsed in 1% acetic acid (30-60 sec) and fixed. Finally, slides were rinsed in running water and stained with Giemsa (Fisher Diagnostics, Orangeburg, NY). Analysis of ^{3}H-thymidine incorporation for autoradiography was done by (1) counting the number of labelled and unlabelled cells, and (2) measuring the percent area of labelling in cells using a PGT digitizer. Background was defined as $\leq$ 25 grains/window on the PGT digitizer at a total magnification of 1550X.

The total incorporation of ^{3}H-thymidine using beta scintillation was determined as follows. Control and growth inhibitory concentrations of BA were added in triplicate 24 h after cell plating. After 54 h, 1 or 2 uCi/ml of ^{3}H thymidine was added for 18 h. Cells were washed with Hank's Balanced Salt Solution (HBSS) without Ca^{++} or Mg^{++} and lifted by trypsin/EDTA. Collected cells were either harvested onto glass filter paper or not filtered. Aqueous scintillation fluor (Packard Instrument Co., Inc., Downers Grove, IL) was added to the filters and to the unfiltered cell suspensions. Counts per minute were determined by liquid scintillation spectrophotometry.

Incorporation of ^{3}H-IdUR by RA-treated Cells

The incorporation of ^{3}H-IdUR was determined by autoradiography and beta scintillation as in the above section with the following exceptions: (1) for both autoradiography and beta scintillation one uCi/ml of ^{3}H-5-Iodo- deoxyuridine (Amersham Inc., Arlington Hts., IL) (specific activity of 5 Ci/mM) was added at 54 h after the start of incubation, (2) the cytostatic concentration of RA was used for both autoradiography and beta scintillation, (3) analysis of incorporation of ^{3}H-IdUR by autoradiography was done only by counting labelled and unlabelled cells, and (4) cellular suspensions were filtered for beta scintillation.

Migration Assay

The methodology in this investigation was a modification of the migration assay of Varani et al. (1978). I utilized either the Transwell chamber system (Costar, Cambridge, MA) or a diffusion chamber (Persky and Grganto, 1987). Transwell chambers have a well within a well design containing a 3 or 5 um diameter pore polycarbonate membrane (10 um in thickness) in the inner well.

Trial 1 In the first investigation, polycarbonate membranes with 3 um pores were utilized. Cells were plated at least in triplicate in the upper wells of the Transwell chambers (70,000 cells/well, 50,000 cells/well and 5,000 cells/well). Cells were allowed to attach to the polycarbonate membranes for 24 h prior to treatment with cytostatic concentrations of RA, BA, colchicine, or taxol. Cells were then incubated for three days without refeeding. Drugs were not replenished during the migration assays. Cytostasis was defined as the

concentration of drug that inhibited cell growth without measurable cell death. The experiment in which cells were treated with or without BA (50,000 cells/well) was quantitated by SEM (n=4, 500X). The percent migration was determined from the number of cells counted/field times 50 (the number of fields counted) times 15.36 (50 fields at 500 X is equal to 1/15.36 of the total surface area). Colchicine, RA, and taxol-treated cells were qualitatively examined.

Trials 2 - 4 In the second investigation, quantification of migration by SEM was done using 5 um diameter pore polycarbonate membranes (Nuclepore, Pleasanton, CA) in diffusion chambers or 5 um pore Transwell chambers. Migration was evaluated by counting cells on the bottom surface of the membranes after 18 (Trial 4) and 72 h (Trials 2 and 3) of incubation. The number of migratory cells was determined by counting cells within 50 fields per membrane, each field at 1,700X. This magnification was selected in order to properly delineate cell boundaries. Percent migration was determined by two different methods. The first method was defined as the number of migratory cells counted per field times 50 (the number of fields counted) times 177.59 (50 fields at 1700X is equal to 1/177.56 of the total surface area) divided by 50,000 (the number of cells originally plated). For the 18 h time period, untreated and BA-treated early passage (less than six passages) cells were plated into Transwell chambers (50,000 cells/well). For the 72 h time period, untreated, BA-treated, BA-pretreated (72 h before plating), RA-treated, and calf serum-treated early passage (Trial 2) cells were plated into either the Transwell system or a diffusion chamber (50,000 cells/well).

The second methodology for determining percent of migration was calculated only for untreated and BA-treated cells by incorporating a proliferation factor, P_f, for cellular proliferation in 35 mm dishes during the 3 day incubation period. The two P_f values were obtained with a hemacytometer by counting the number of untreated and BA-treated cells after 3 days of incubation. The P_f values were 777,275 and 177,500 for untreated and BA-treated cells, respectively. This method was thus defined as the number of cells per 1,700X field times 50 divided by P_f.

Trial 3 Quantification of migration was done on later passage B16a cells (greater than 6 passages) . Untreated, BA-treated, and BA-pretreated (72 h before plating) cells were plated into a Transwell chamber (n=4, 50,000 cells/well).

Statistics

Students' t tests were performed for the results of all experiments except for the data from trials two and three of the migration assays where Welch's tw (t') unequal variance or Bechhofer-Dunnett-Krishnaiah-Armitage equal variance multiple comparison tests were done (Wilcox, 1987).

Scanning Electron Microscopy

All membranes were prepared according to standard techniques except that the membranes were air dryed instead of critical point dryed. Briefly, membranes were washed in 0.2M cacodylate buffer, fixed in 2% glutaraldehyde, and post fixed in 1% osmium tetroxide. The membranes were dehydrated, air dryed, mounted, and sputter coated with gold (27 nm coating; Polaron ES100 Series II sputter coater).

Membranes were viewed with a JEOL JSM-840A scanning electron microscope.

Transmission Electron Microscopy

A preliminary ultrastructural study was undertaken to provide evidence that RA and BA modify the cytoskeleton of B16a cells. The transmission electron microscopy (TEM) was done according to the method of McDonald (1984) and Costar Corporation (personal communication) in order to preserve microfilaments. Briefly, a migration assay was run with untreated, RA-pretreated, and BA pretreated cells in Transwell chambers. All steps were run with membranes still in the Transwell chambers. Membranes were fixed after 72 h with 2% glutaraldehyde in 50 mM cacodylate buffer (pH=7.4) containing 5 mM $CaCl_2$ for 30 min at 37°C. The membranes were rinsed in buffer (3X) for 5 min at 37°C and incubated in buffer containing 0.8% $K_3Fe(CN)_6$ for 30 min. The membranes were incubated in buffer containing 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$ for 30 min, rinsed in buffer, and then rinsed in distilled water (3X). The membranes were stained en bloc with 2% uranyl acetate (distilled water) for 60 to 120 min, rinsed in distilled water (3X) and then dehydrated in grades of ethanol. Membranes were infiltrated into resin (Epon-Araldite), cut out of the Transwell inner well, and polymerized in blocks at 60°C for several days. Thin sections were cut, stained in 1% uranyl acetate (in 70% methanol) and lead citrate, and viewed with a Hitachi H-600 transmission electron microscope.

RESULTS

Growth Curves

In vitro growth curves of BA (Fig. 1), RA (Fig. 2), and colchicine-treated (Fig. 3) cells were determined by hemacytometer. Cell viability for the six different treatment groups (control medium, control medium + 0.1% ethanol, RA, CS, BA, and colchicine) as determined by the trypan blue exclusion test was always greater than 90%.

Cells were treated for 72 h with different concentrations of BA (Fig. 1). A concentration of 2 mM BA was found to be cytotoxic as fewer cells were found after 72 h (130,500 $\pm$ 33,600) than were plated (150,000). A concentration of 1.5 mM BA was not cytotoxic yet inhibited proliferation by 77% $\pm$ 6% as compared to the controls (0 mM). After 72 h of incubation in 1.5 mM BA, viability was comparable to 72 h controls (0 mM), i.e., 97.3% $\pm$ 1.5% and 98.9% $\pm$ 0.3%, respectively. There was a range of cytostatic concentrations between 0.5 mM and 1.5 mM that did not cause cytotoxicity. However, the 'cytostatic' concentration of 1.5 mM BA was chosen to be used for all further experiments. It was also observed that the lowest concentration of BA (0.1 mM) inhibited proliferation by 35%.

The cytotoxic/cytostatic concentrations for RA were determined to be 1×10^{-5} M/1×10^{-6} M, respectively (Fig. 2). The cytostatic concentration was a range of values between 10^{-6} and 10^{-7} M. The 1×10^{-6} concentration of RA inhibited proliferation as compared to the 0.1% ethanol control by 65% and was used for all further cell

proliferation and migration experiments. The lowest concentration tested (1×10^{-8} M) inhibited proliferation by 50%. The dishes that contained 1×10^{-4} M concentration of RA were found to have more viable cells than 1×10^{-7} M. Since crystals of RA were found in the 1×10^{-4} M concentration medium, I believe that this concentration produced a supersaturated condition such that the RA was not able to completely dissolve in ethanol. The addition of 0.1% ethanol did not affect proliferation ($p<0.05$) (Table 1). Proliferation was significantly inhibited ($p<0.001$) by 59.1% when 10% CS was substituted for 10% fetal calf serum (Table 1).

Figure 3 is the growth curve of colchicine-treated cells. The cytostatic concentration was determined to be 5×10^{-7} M.

^{3}H-Thymidine Incorporation in BA-treated Cells

Inhibition of ^{3}H-thymidine incorporation by BA was determined by autoradiography and beta scintillation counting. In the experiments using autoradiography, two different ^{3}H-thymidine incubation times, 18 h and 48 h, were used. Two separate experiments were done in which the cells were labelled for 18 h, starting 54 h after cell plating. Unlabelled and labelled cells were counted from twelve different fields (average of 125 cells counted/field, 200X) from three different slides (two slides from the first 18 h protocol, one from the second 18 h protocol). Autoradiography revealed that after 18 h 59.1% (SEM = 9.3) of the untreated cells had incorporated the ^{3}H-thymidine, while only 32.9% (SEM = 0.6%) of the BA-treated cells had incorporated the label. The percent inhibition of ^{3}H-thymidine incorporation was calculated to be 42.0% (SEM = 7.1%) ($p<0.001$). Figure 4a and 4b illustrate two

autoradiographs of BA-treated cells that were (Fig. 4A) or were not treated with ^{3}H-thymidine for 18 hr. Three of nine cells in Fig. 4A were interpreted to be labelled.

Two autoradiography methods (A and B) were used for counting cells that were labelled with ^{3}H-thymidine for 48 h. For both methods, cells were labelled 24 h after plating. For method A, the absolute number of labelled and unlabelled cells was counted. After 48 h of incubation, 96% of the untreated cells were labelled, while 58.5% of the BA-treated cells were labelled (data not shown). The percent inhibition of incorporation of ^{3}H thymidine was calculated to be 39%. For method B, the amount of labelling was calculated on a PGT digitizer from the percent of a set area containing the label (739.8 um^{2}, 689X). A total of 53 cells were digitized for each group, i.e., untreated and BA-treated cells. A minimum of 10 random fields were investigated for each group. The area labelled in untreated and BA-treated cells was calculated to be 6.6% (S.D= 3.2) and 3.2% (S.D.=3.7), respectively. The percent areas were significantly different ($p>0.0001$).

Lastly, ^{3}H-thymidine incorporation for untreated and BA-treated cells was measured after an 18 h incubation period by beta scintillation. The average percent inhibition of ^{3}H-thymidine incorporation was 80% ($p<0.001$) (Table 2).

^{3}H-IdUR Incorporation in RA-treated Cells

Inhibition of ^{3}H-IdUR incorporation by RA was determined by autoradiography and beta scintillation counting. For the autoradiography, unlabelled and labelled cells were counted from 21 (untreated) or 24 (RA-treated) different fields (200X) from three

different slides. Autoradiography revealed that 64.5% (SEM=1.6) of the untreated cells were labelled after 18 h incubation with ^{3}H-IdUR, while only 29.8% (SEM=1.5) of the RA-treated cells had incorporated the label. The percent inhibition of ^{3}H-IdUR was calculated to be 53%. For the beta scintillation, the percent inhibition of ^{3}H-IdUR incorporation was 72% ($p<0.0001$) (Table 3).

Migration Assay

The initial experiments using 3 um diameter pore polycarbonate membranes led to further analysis and quantification of migration with 5 um diameter pore membranes. Light microscopy showed untreated cells grown on plastic to be heterogeneous in morphology (round to flat) as well as diverse in cell size. In contrast, BA-treated cells were a homogeneous population of large, flattened cells.

The morphology of untreated cells (Figs. 5 and 6), RA-treated (Fig. 7), BA-treated (Fig. 8), colchicine-treated (Fig. 9) and taxol-treated (Fig. 10) cells was also evaluated by SEM on both 3 and 5 um diameter pore polycarbonate membranes. In all cases, cells on the top surface of the polycarbonate membranes had morphology similar to comparable treated cells grown on plastic. Butyric acid (Fig. 8), colchicine (Fig. 9), and taxol (Fig. 10) treatment induced a flattened morphology as compared to untreated cells whether the cells were grown on plastic or on polycarbonate membranes. Colchicine and taxol-treated B16a cells on top of the membrane were not as flattened in shape as BA-treated cells. The majority of colchicine (Fig. 9) and taxol treated cells (Fig. 10) were covered with blebs. Retinoic acid induced a spindle-shaped morphology. The major observation was that many more

BA-treated cells migrated through 3 um pore membranes than untreated, RA, colchicine, or taxol-treated cells. The experiment in which cells were treated with or without BA (50,000 cells/well) was quantitated using SEM. The increase in the percent migration induced by BA over untreated controls was calculated to be 511% (Trial 1, Table 4). If migration rates were formulated to incorporate proliferation during a three day incubation period (Fig. 1), BA enhanced migration by 2300%. Migration through the membrane for untreated (Fig. 5), RA-treated, colchicine (Fig. 9) or taxol (Fig. 10) was < 2% of the original plated number. Cell plating density, which ranged from 5,000 to 75,000 cells per well, did not have an effect on the migration of untreated and BA-treated cells through the 3 um pore polycarbonate membrane (data not shown).

Later experiments quantitated by SEM the migration of untreated and treated cells through 5 um pore diameter membranes after 18 (Trial 4, Table 4) and 72 hrs (Trials 2 and 3, Table 4) of incubation. Both diffusion chambers and Transwell chambers were used. Table 4 illustrates that Transwell chambers were used for Trials 3, 4, and PBA, RA, CS treatments of Trial 2. Diffusion chambers were used for the other parameters of Trial 2.

Trial 2. Using early passage cells (passage less than six), an average of 3.11 control cells/field (SEM=1.22) and 3.23 BA-treated cells/field (SEM=1.13) were observed on the bottom of the membrane (Table 4). The number of cells per field initially suggest no significant difference in migration between untreated and BA-treated cells. However, these numbers do not take into account the partial

cytostasis induced by BA. If migration rates were formulated to incorporate cellular proliferation (Fig. 1) during a three day incubation period, BA-treatment enhanced migration by 456% over controls ($p< 0.05$) (Table 5). The migration of BA-treated cells using 10% FCS was 192% greater than untreated cells using 10% calf serum ($p< 0.05$) (Trial 2, Table 4). A further enhancement of migration was induced by BA if cells were pretreated for 72 h with BA before plating. Pretreatment increased migration over non-pretreated BA-treated cells by 196% ($p<0.05$) and similarly increased migration over untreated cells by 203% ($p<0.05$) (Trial 2, Table 4). If 72 h migration rates were formulated to incorporate proliferation, the rate for the BA pretreated cells was 893% ($p< 0.05$) greater than untreated cells (Table 5). There was no significant difference in migration in the 18 h incubation period between untreated and BA-treated cells (Trial 4, Table 4). In contrast, the migration of RA-treated cells during the 72 h incubation period was decreased by 75% ($p<0.05$) as compared to untreated cells. (Trial 2, Table 4). If 72 h migration rates were formulated to incorporate proliferation, the rate for the RA-treated cells was not significantly different from the untreated cells (Table 5). There was no significant difference in migration rates between 10% CS-treated cells and 10% FCS-treated (CO) cells.

Trial 3. Migration was quantified using later passage B16a cells. The methodology above was used to quantitate migration of untreated, BA-treated, and PBA-treated cells ($n=4$). The percent migration of untreated cells was calculated to be 3.72% of the plated number. Again, I found that BA or PBA increased migration by 438% and 919%,

respectively, over the untreated controls ($p<0.05$, $p<0.05$). If 72 h migration rates were formulated to incorporate proliferation, the rate for the BA-treated late passage cells was 1900% ($p<0.05$) greater and the rate for the BA-pretreated late passage cells was 4000% ($p<0.05$) greater than untreated late passage cells (Table 5).

Transmission Electron Microscopy.

The analysis of the cytoskeleton of untreated, RA-treated and BA-treated B16a cells was a qualitative TEM study. Analysis of untreated B16a cells found two distinct cytoskeletal phenotypes. Cells that were not visibly attached to the substrate displayed few microtubules and intermediate filaments. Microfilaments were visible only along cell margins. Cells that were attached to the substrate displayed a disorganized microtubule and intermediate filament system. Microfilaments were found in the cortical areas of the cytoplasm (Fig. 10). In contrast to untreated cells, RA and BA treatment altered the structure of the cytoskeleton. Retinoic acid-treated cells displayed parallel arrays of microtubules and intermediate filaments (Fig. 11). Less cortical microfilaments were seen with wider focal adhesion contacts than in untreated cells. Butyric acid-treated cells also displayed parallel arrays of microtubules and intermediate filaments oriented along the longitudinal axis of the cells. An extensive cortical microfilament network was seen (Fig. 12). This preliminary ultrastructural study supports the evidence presented in the literature that RA and BA alter the structure of the cytoskeleton.

DISCUSSION

In this investigation, BA enhanced while RA decreased the migration of B16a cells through 3 and 5 um diameter pores. Migration of untreated and RA-treated cells through 3 um pore membranes was negligible (< 2% of cells plated). In contrast to untreated cells, treatment with BA resulted in a 511% increase in migration through 3 um diameter pores. The 3 um diameter pore is near the minimum 1-2 um pore size that allows passage of B16 cells (Tullberg and Burger, 1985), although there are more 3 um diameter pores per membrane than in 5 um or 8 um diameter pore membranes (Costar Co., personal communication). Untreated early passage (<6 passages) cells migrated significantly through 5 um diameter pores (55.1%). The effect of BA on the percent change of migration through 5 um pores was not as pronounced as through 3 um diameter pores (104% vs 511% migration, respectively). On the other hand, RA-treated cells migrated through 5 um pores at 14.0%, i.e., 75% less than untreated cells.

It was important to determine the cytotoxic/cytostatic concentrations of RA and BA since they vary from cell line to cell line (Reese et al., 1985; Kyritsis et al., 1984; Lotan and Nicolson, 1979). The use of non-cytotoxic drug concentrations in in vitro migration assays is important since cytotoxicity will mask enhancement or inhibition of migration (Mareel and DeMets, 1984). I used hemacytometer counts to demonstrate that RA and BA decreased proliferation during the three day incubation period. I used autoradiography and beta scintillation counting to show that RA and BA

also inhibited the incorporation of ^{3}H-thymidine and ^{3}H-IdUR. Both RA and BA have been shown to inhibit proliferation in various tumor cell lines in the G1 phase of the cell cycle (Roberts and Sporn, 1984; Thorgeirsson et al., 1984).

In my study, phase microscopy and SEM revealed morphological changes in RA and BA-treated cells. How these changes are related to migration is not known. The untreated cell population on top of 3 and 5 um pore membranes is heterogeneous (Figs. 4 and 5a). There are, however, more flattened cells on top of 3 um pore membranes than 5 um pore membranes. Concomitantly, bottom surfaces of 5 um pore membranes are dominated by flattened cells. I concluded that increasing the pore size from 3 to 5 um allowed more flattened cells to migrate to the bottom surface. Rounded-up cells, i.e., mitotic cells, do not migrate. It is therefore not surprising that incubation with BA, which enhances cell flattening, increases cell migration. The morphology of RA-treated cells differs from either untreated or BA-treated cells. The RA-treated cell population consisted of spindle to flattened shaped cells.

Various methods have been used to quantify motility (Volk et al., 1984; Keller and Zimmermann, 1986), deformabilty (Ochalek et al., 1988) and to correlate these properties to metastatic potential. Methods used to quantify motility include time lapse photography (Keller and Zimmermann, 1986) and the phagokinetic assay (Volk et al., 1984). Data from the above experiments have in some cases been found to be in direct conflict with data from migration assays (Spiro and Mundy, 1980). Our experimental protocol emphasizes that migration

incorporates both active cellular deformabilty and motility (locomotion) (Daughaday et al., 1981). Migration is therefore defined as the net summation of cellular deformability and motility. Microtubule (MT) inhibitors, e.g., colchicine, and microfilament (MF) disrupting agents, e.g., cytochalasin B, inhibited the migration of tumor cells in a micropore filter system (Spiro and Mundy, 1980) even though cell motility was enhanced (Keller and Zimmermann, 1986). The decreased migration seen with microtubule inhibitors may be due to reduced cellular deformability (Ochalek et al., 1988). Retinoic acid is not a microtubule inhibitor, so the decrease in migration of RA-treated cells may be due to affecting motility rather than deformability. In contrast, since the percent increase in migration of early passage BA-treated cells through the 3 um diameter pore membrane is greater than through the 5 um pore membrane, BA may be affecting deformability rather than motility.

Retinoic acid and BA are being investigated as potential antitumor-antimetastatic agents. Studies on various cell lines (Roberts and Sporn, 1984; Thorgeirsson et al., 1984; Ryan et al., 1987), including the B16 melanoma cell line (Lotan and Nicolson, 1979; Nordenberg et al., 1986) have stressed the effect of RA and BA on growth inhibition and differentiation. The change in morphology in these cell lines has been linked to a change in the cytoskeleton (Lehtonen et al., 1983; Ng et al., 1985; Altenburg et al., 1976; Borenfreund et al., 1980).

The role of the cytoskeleton in migration has been demonstrated by microtubule inhibitors (e.g., colchicine, vinblastine) and

microfilament inhibitors (e.g., cytochalasin B) (Spiro and Mundy 1980). These inhibitors reduce migration. Both RA and BA have been shown in various tumor cell lines to increase the formation of microfilaments and vinculin plaques, and to reorganize vimentin intermediate filaments (Lehtonen et al., 1983; Ng et al., 1985; Altenburg et al., 1976; Borenfreund et al., 1980). I also observed by TEM an increase in microtubule and microfilament organization in Bl6a cells. Retinoic acid and BA induced changes in the cytoskeleton appear to be similar, although the surface morphology and shape of RA or BA-treated cells differ as examined by phase contrast microscopy and SEM. Subtle changes in the cytoskeleton may induce migration of BA-treated cells while inhibiting the migration of RA-treated cells. Butyric acid may have a role in increasing the amount of acetylated a-tubulin (Grant et al., 1987) thus contributing to the stabilization of microtubules (Piperno et al., 1987).

The role of cellular migration in invasion and metastasis needs further investigation. The Transwell system has merit for both migration and invasion assays. Various investigators have studied invasion by using porous polycarbonate membranes coated with a reconstituted basement membrane (Matrigel) (Albini et al., 1987; Hendrix et al., 1987; Repesh, 1989). An increase in migration of tumor cells <u>in vitro</u> has been linked to enhanced invasion <u>in vitro</u> (Grimstad, 1987; Verschueren et al., 1988). However, enhanced <u>in vitro</u> migration of tumor cells may not necessarily lead to increased metastasis. Grimstad (1988) found that hypermotile selected fibrosarcoma cells were less metastatic than the original parent population. Mohler et al.

(1988) found, in contrast, a positive correlation between metastatic potential of Dunning R-3327 rat prostatic adenocarcinoma cells and motility. Invasive cells that form specialized pseudopodia have been interpreted by Kramer et al. (1986) to form adhesion contacts with the matrix. I observed that migratory cells (e.g., Fig. 5b) also form pseudopodia when passing through 5 um diameter pores. In addition, I have observed by TEM focal adhesion contacts (not illustrated).

Cell motility has been linked to various parts of the cell cycle in 3T3 fibroblasts (Thurston and Palcic, 1987). Thurston and Palcic (1987) found a peak in the rate of cell movement in G2 and a significant increase in the average rate of movement as cells transit from G1 to S phase. The significance of their data, which was obtained on 3T3 cells, as applied to a tumor cell system is not known. Since RA and BA block entry into the S phase of the cell cycle (Roberts and Sporn, 1984; Thorgeirsson et al., 1984), Thurston and Palcic's data may not be applicable. Thurston and Palcic (1987) also found a weak correlation between the rate of cell motility and the rate of cell proliferation. It is extremely important to emphasize that a decrease in proliferation is not sufficient to explain an increase in migration of BA-treated cells. A 5×10^{-5} M concentration of RA inhibits both proliferation and migration. Calf serum (CS) does not have as many of the growth factors that are in fetal calf serum. Calf serum was found to inhibit cell proliferation by 59.1% as compared to 10% FCS (Table 1). However, the migration of CS-treated cells was not significantly decreased from the untreated and BA-treated cells. The migration of calf serum-treated cells, however, is significantly greater than the

RA-treated cells ($P<0.05$). Another method of evaluating the migration data is the use of a proliferation factor. If the proliferation factor is applied to migration rates of RA-treated cells, there is no significant decrease in migration. Therefore, I conclude that the decrease in migration produced by RA was due to both an inhibitory effect on cell migration and a decrease in proliferation. A purely speculative explanation for the anti-migratory effect of RA is that changes in glycoproteins found on the cell surface may influence migration (Lotan et al., 1987 a,b; Couch et al., 1987, 1988).

The migration rates for untreated and BA-treated early passage cells were similar (Table 4). However, if the decrease of cellular proliferation by BA is included in defining migration, the enhancement of migration by BA is significant (Table 5). Hendrix et al. (1985) have stated that cells which invaded the amnion had similar proliferation rates as cells which did not invade. The observation that migration by preincubated BA-treated cells was significantly different from untreated and BA-treated cells suggests that further changes occur in the phenotype of these cells after 3 days.

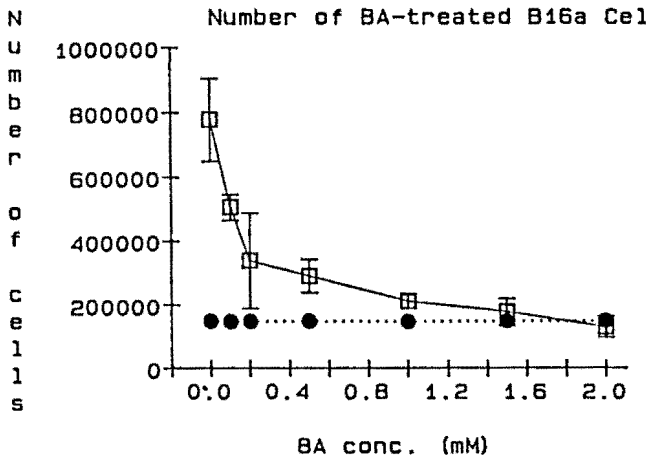
Tumor cell migration has been investigated in vitro using short time periods (4-18 h) (Varani et al., 1985; Nabeshima et al., 1986; Grimstad, 1988). It is questionable whether four hours is long enough to quantitate migration in this or any other assay. Perhaps such a short period does not adequately reflect the in vivo condition. I noted that there was no significant difference between the migration of untreated and BA-treated cells at 18 h (even if 18 h proliferation data were used in the calculations, data not shown). Cells treated for 72 h

with BA before plating show a significant increase in migration (greater than both the untreated and BA-treated cells) in a three day assay (Tables 4 and 5). It would be of interest to pretreat cells with BA or RA for 72 h prior to an 18 h migration assay. This project would make unnecessary the use of a proliferation factor in comparing migration rates.

In summary, RA and BA warranted investigation for two main reasons. First, these two drugs can be less host toxic and less mutagenic than conventional chemotherapeutic drugs when administered at cytostatic concentrations. Second, the effects of RA and BA on the individual steps of the metastatic cascade are only beginning to be known.

Figures 1-3.

Dose response curves as measured by hemacytometer for BA, RA, and colchicine-treated B16a cells. Cells were plated (50,000 cells/ml, 3 ml total) in culture dishes. Various concentrations of BA, RA, and colchicine were added after 24 hrs. Cell number was determined 96 hrs after plating. Dotted line indicates number of cells plated.


Number of BA-treated B16a Cells
Number of cells
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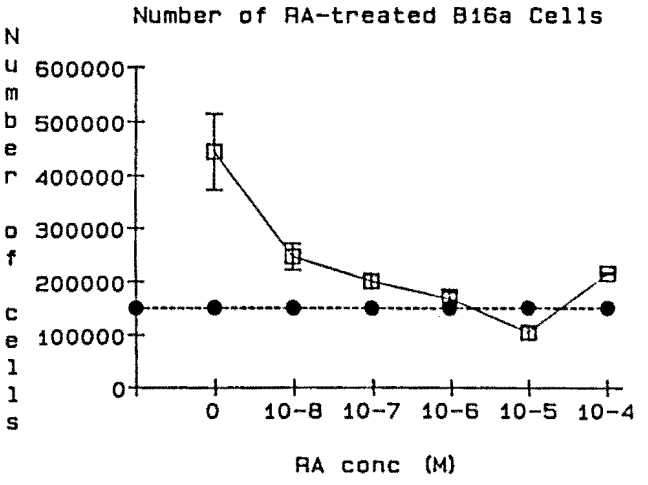
800000
600000
400000
200000
0
0.0
0.4
0.8
1.2
1.6
2.0
BA conc. (mM)


Number of RA-treated B16a Cells
Number of cells
600000
500000
400000
300000
200000
100000
0
0
10-8
10-7
10-6
10-5
10-4
RA conc (M)

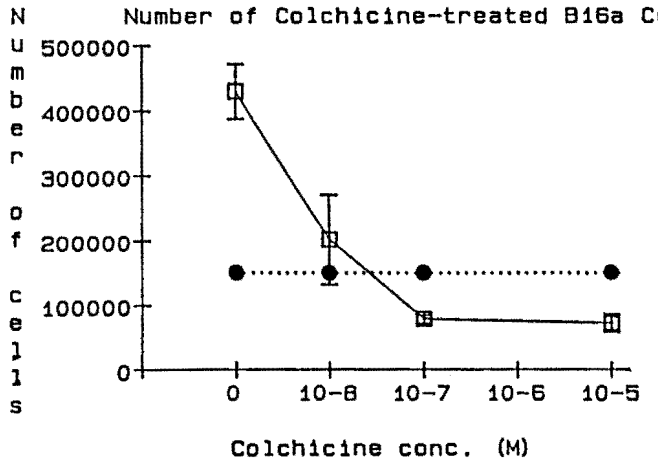

Number of Colchicine-treated B16a Cell
Number of cells
500000
400000
300000
200000
100000
0
0
10-8
10-7
10-6
10-5
Colchicine conc. (M)

Figure 4.

Two autoradiographs of BA-treated cells that were (Fig. 4A) or were not (Fig. 4B) treated with ^{3}H-thymidine for 18 hr. The number of grains over all nuclei were counted by two independent investigators. For Fig. 4A, the mean $\pm$ SE of the number of grains over the nuclei of the six cells that "appeared" unlabelled was 10 $\pm$ 0.7 grains. The three nuclei that "appeared" contained > 40 grains per nuclei. For Fig. 4B, an average of 13.3 $\pm$ 1.1 grains were counted for the 10 nuclei. By comparing Fig 4A to 4B, three of the nine cells in Fig. 4A were interpreted to be labelled cells (arrows).

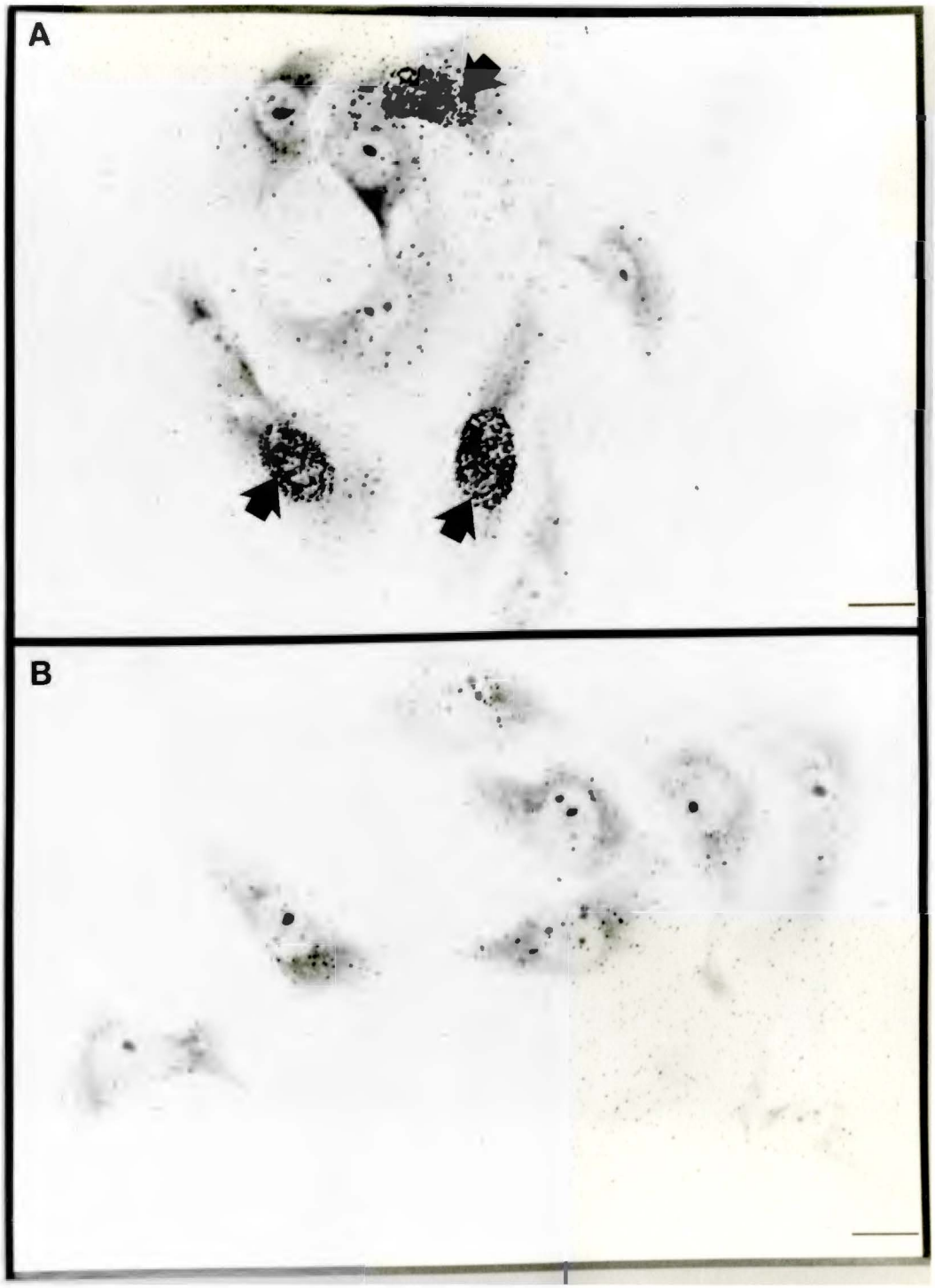

A
B

Figure 5.

Scanning micrograph of untreated cell on the top surface of a 3 um pore polycarbonate membrane after 72 hrs of incubation. (Bar = 10 um)

Figures 6-8.

Scanning micrographs of untreated (Fig. 5), RA-treated (Fig. 6), and BA-pretreated (Fig. 7) cells on the top ("a") and bottom ("b") surfaces of 5 um diameter pore polycarbonate membranes after 72 hrs of incubation. Note the pseudopod coming out of a pore in Figures 5 and 6 (arrow). (Bar = 10 um)

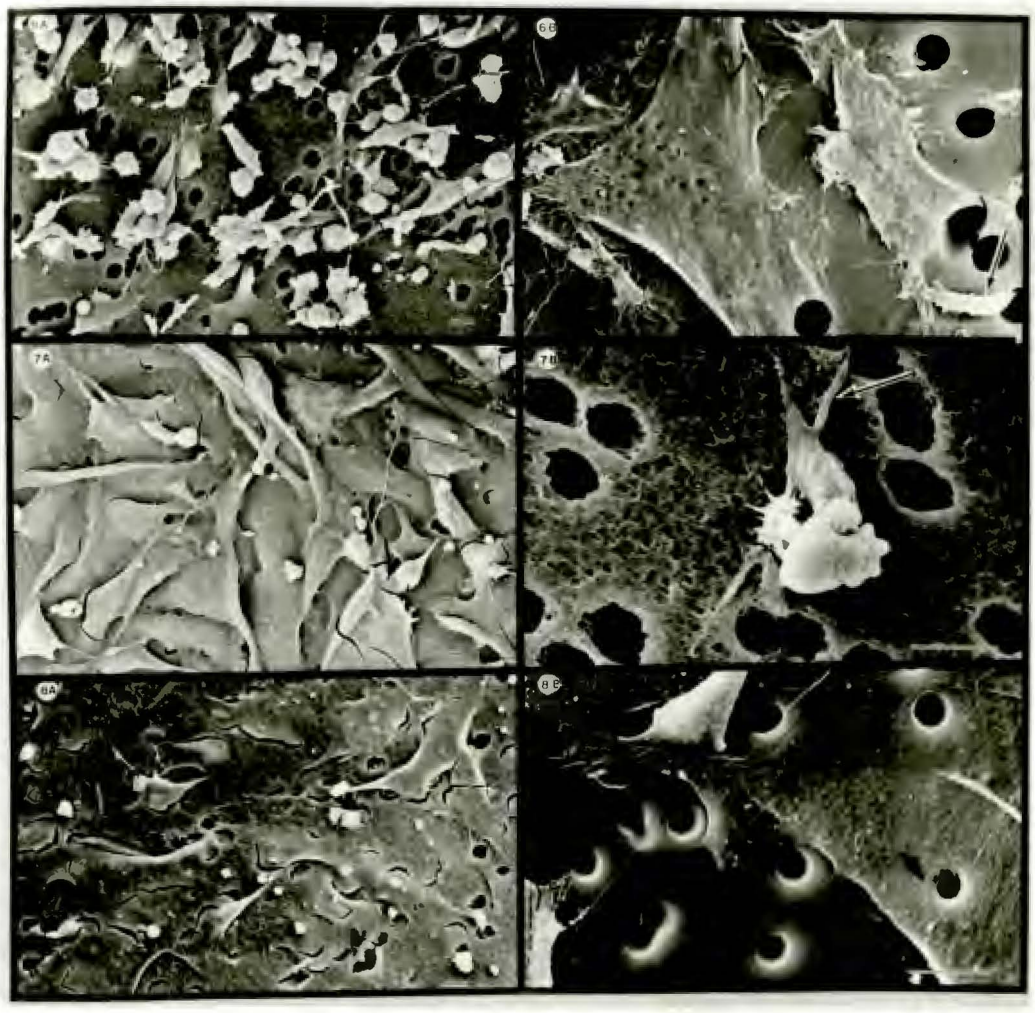

6A
6B
7A
7B
8A
8B

Figure 9-10.

Scanning micrographs of colchicine and taxol-treated cells on the top surface of 5 um diameter pore polycarbonate membranes after 72 hrs of incubation. (Bar = 10 um)

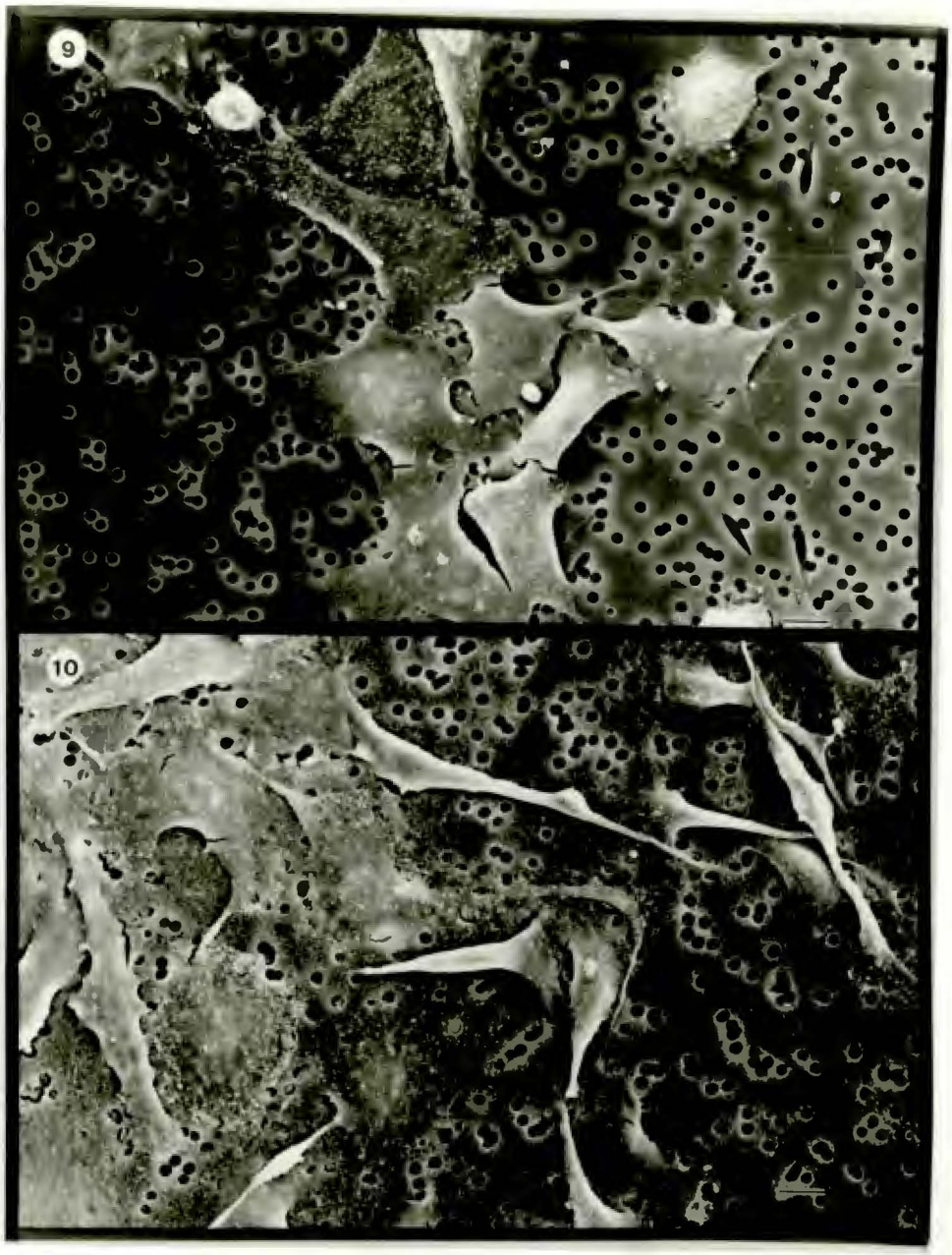

9
10

Figure 11-13.

Transmission micrographs of untreated (Fig. 11), RA-treated (Fig. 12), and BA-treated cells (Fig. 13). The untreated cell (Fig. 11) displays a disorganized microtubule and intermediate filament system with microfilaments in the cortical area of the cytoplasm. The RA-treated cell (Fig. 12) shows an organized microtubule and intermediate filament system with less cortical actin than untreated cells. The BA-treated cell (Fig. 13) displays an extensive system of parallel microtubules and a large amount of cortical actin. Microtubules (black arrow), intermediate filaments (open arrow), and microfilaments (arrow head) are present in all three cell types (Bar = 0.2 um).

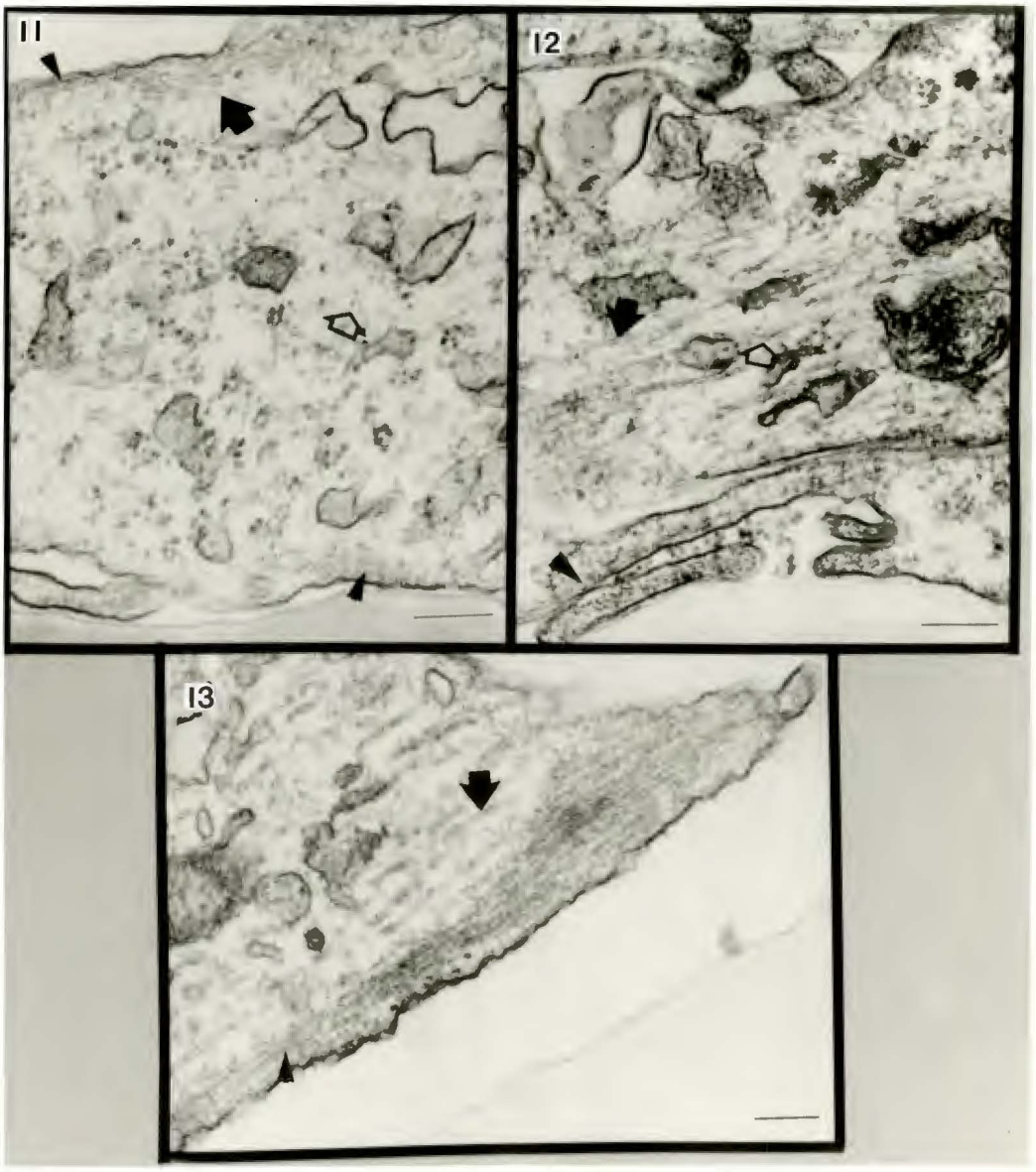

I1
I2
I3

TABLE 1.

The means of the untreated and 0.1% ethanol groups were not significantly different ($p > 0.05$). The means of the hemacytometer counts for 10% FCS-treated cells and 10% CS-treated cells were significantly different ($p < 0.001$).

NUMBER OF CELLS AFTER 72 HOURS OF INCUBATION

AS DETERMINED BY HEMACYTOMETER

	UNTREATED	0.1% ETHANOL	10% FCS	10% CS
Mean	4.8×10^5	4.5×10^5	4.3×10^5	1.8×10^5
St. Dev.	4.3×10^4	6.8×10^4	4.2×10^4	2.1×10^4
N	4	3	3	3

TABLE 2.

The ^{3}H-thymidine incorporation by untreated and BA-treated cells after 18hr incubation was measured. Cells were lifted with trypsin/EDTA and the cell suspension was either unfiltered or filtered with a cell harvester. The percent inhibition by BA of ^{3}H-thymidine incorporation was 67% for unfiltered cell suspensions and 93% for filtered suspensions. The average percent inhibition was 80%.

INCORPORATION OF ^{3}H-THYMIDINE

CPM AS DETERMINED BY BETA SCINTILLATION

	UNFILTERED CON	UNFILTERED BA	FILTERED CON	FILTERED BA
	1 uCi/ul		2 uCi/ul	
Mean	6.3×10^{5}	2.0×10^{5}	1.5×10^{6}	1.0×10^{5}
St Dev	2.7×10^{4}	7.6×10^{4}	1.4×10^{5}	2.7×10^{4}
N	3	3	3	3

TABLE 3.

The ^{3}H-IdUR incorporation by untreated and RA-treated cells after 18 hr incubation was measured. Cells were lifted with trypsin/EDTA and the cell suspension was filtered with a cell harvester. The percent inhibition of ^{3}H-IdUR incorporation by RA was 72%.

INCORPORATION OF ^{3}H-IdUR

CPM AS DETERMINED BY BETA SCINTILLATION

	FILTERED CONTROL	FILTERED RA
Mean	1.9×10^{4}	5.2×10^{3}
St Dev	2.4×10^{3}	1.0×10^{3}
N	3	6

TABLE 4.

The migration of B16a cells through 3 um and 5 um diameter pore polycarbonate membranes. There was a significant difference in the migration through a 3 um diameter pore membrane between untreated and BA-treated cells ($p < 0.013$) (Trial 1). In contrast, there was no significant difference between untreated and BA-treated early passage cells (Trial 2), but there was a significant difference between untreated and BA-treated late passage cells after 72 h incubation (Trial 3). Interestingly, there was a significant increase in migration of BA-pretreated early passage cells as compared to control and BA-treated cells ($p < 0.05$, $p < 0.05$, respectively, Trial 2). There was also a significant decrease in migration of RA-treated cells as compared to control and calf serum-treated cells ($p < 0.05$, $p < 0.05$, respectively). Lastly, there no was a significant difference between untreated and BA-treated cells after 18 h incubation. This table includes percent migration and percent change in migration. The percent change in migration of untreated cells was considered to be 100%.

TABLE IV

TRIAL	TIME	MEMBRANE PORE SIZE	TREATMENT	N	MAGNIFICATION	MEAN # OF CELLS (± SE)	% MIGRATION	% CHANGE IN MIGRATION	SIGNIFICANCE LEVEL
#1	72 hr	3 µm	Control	4	500x	0.82 ± 0.02	1.26	--	--
	72 hr	3 µm	BA	4	500x	4.19 ± 0.62	6.42	+411	0.013
EARLY PASSAGE									
#2	72 hr	5 µm	Control	5	1700x*	3.11 ± 0.89	55.13	--	--
	72 hr	5 µm	BA	6	1700x*	3.23 ± 0.72	57.26	+4	**
	72 hr	5 µm	PBA	5	1700x*	6.32 ± 0.19	74.69	+203	0.05
	72 hr	5 µm	RA	5	1700x*	0.79 ± 0.15	14.00	-75	0.05
	72 hr	5 µm	CS	5	1700x*	1.68 ± 0.18	29.78	-54	N.S.***
LATE PASSAGE									
#3	72 hr	5 µm	Control	4	1700x*	0.21 ± 0.04	3.72	--	--
	72 hr	5 µm	BA	4	1700x*	0.92 ± 0.17	16.31	338	0.05
	72 hr	5 µm	PBA	4	1700x*	1.93 ± 0.23	35.68	819	0.05
EARLY PASSAGE									
#4	18 hr	5 µm	Control	3	1700x*	1.07 ± 0.21	18.97	--	--
	18 hr	5 µm	BA	3	1700x*	1.16 ± 0.30	20.56	8	N.S.***

* The 1700x magnification was used to delineate cell boundaries. Care was taken not to overlap between cells.

** Refer to proliferation table, Table V.

*** N.S. = not significant.

TABLE 5.

The migration rate of control, BA-treated, BA-pretreated and RA-treated cells after 72 hr incubation with a proliferation factor incorporated into the migration rate (data obtained from trials 2 and 3, Table IV). Note that early an late passage cells were used for BA experiments while only early passage cells were used for the RA experiments. The migration value was defined as the number of the cells per field times 50 divided by the final hemacytometer cell population after 72 hr incubation (Fig. 1). There was a significant increase in migration of BA-treated and BA-pretreated cells as compared to control cells ($p < 0.05$, $p < 0.05$, respectively). Retinoic acid treatment did not significantly change migration rates.

MIGRATION VALUES

(INCLUDING THE PROLIFERATION RATE)

	CONTROL (CO)	BUTYRATE (BA)	PRE-BUTYRATE (PBA)
N	5	6	5
MEAN	1.99×10^{-4}	9.08×10^{-4}	17.8×10^{-4}
(EARLY PASSAGE)			
ST DEV	1.26×10^{-4}	4.95×10^{-4}	1.27×10^{-4}
STEM	0.56×10^{-4}	0.20×10^{-4}	0.06×10^{-4}
% MIG.	---	456*	893*
N	4	4	4
MEAN	1.36×10^{-5}	2.58×10^{-4}	5.44×10^{-4}
(LATE PASSAGE)			
ST DEV	0.51×10^{-5}	0.96×10^{-4}	1.28×10^{-4}
STEM	0.26×10^{-5}	0.48×10^{-4}	0.64×10^{-4}
% MIG.	---	1897*	4000*

	CONTROL (CO)	RETINOIC ACID (RA)
N	5	5
MEAN	1.99×10^{-4}	2.36×10^{-4}
ST DEV	1.26×10^{-4}	1.27×10^{-4}
STEM	0.56×10^{-4}	0.45×10^{-4}
% MIG	---	118

* SIGNIFICANTLY DIFFERENT FROM THE CONTROL ($P < 0.05$)

CHAPTER V

THE EFFECT OF BUTYRIC ACID AND RETINOIC ACID ON INVASION AND EXPERIMENTAL METASTASIS OF MURINE MELANOMA CELLS

ABSTRACT

The effect of butyric acid (BA) and all trans-retinoic acid (RA) on murine melanoma cells was investigated in vitro and in vivo. The in vitro assays included ^{3}H-IdUR incorporation, adhesion, migration and invasion experiments. Butyric acid decreased ^{3}H-IdUR cellular incorporation within 24 hrs and increased adhesion as measured by trypsin release of ^{3}H-IdUR labelled cells from either polycarbonate (p.c.) or Matrigel-coated p.c. membranes. Migration and invasion rates after 72 hrs were quantified by scanning electron microscopy (SEM). The invasion barrier consisted of Matrigel coated p.c. membranes. Butyric acid significantly enhanced migration and invasion of B16a cells, while RA significantly decreased migration and invasion B16a and K-1735 cells. Subcutaneous administration of either BA or RA pellets significantly decreased the number of lung nodules in the experimental metastatic assay. The experimental metastatic assay is defined as a tail vein inoculation protocol followed by subsequent lung evaluation.

INTRODUCTION

The role of differentiating agents in the treatment of neoplasms and metastases is becoming increasingly important. Since differentiating agents may be less host-toxic and less mutagenic than chemotherapy, they may be used between rounds of chemotherapy to help prevent regrowth and diversification of remaining tumor cells (Nicolson and Custead, 1985). One such agent, 13-cis-RA (topically applied) has produced partial responses in patients with melanoma (Lippman et al., 1987).

In vitro pretreatment of tumor cells with differentiation agents such as BA (Takenaga, 1986), dimethyl sulfoxide (DMSO) (Takenaga, 1984), and N-methylformamide (NMF) (Tofilon et al., 1986) has increased *in vivo* experimental metastasis. In addition, *in vitro* pretreatment with both RA and dibutyl cyclic AMP together has changed the pattern of organ metastasis of F9 embryonal carcinoma cells (Terrana et al., 1986).

Few studies have investigated the *in vivo* effect of differentiation agents on metastasis without drug pretreatment. Experimental evidence indicates that both the route and timing of the administration of differentiation agents is critical. N-methylformamide, when given prior to tail vein injection of tumor cells, increased experimental metastases, while NMF given after tail vein injection decreased experimental metastases (Tofilon et al., 1986). Dietary retinoids given to athymic mice decreased the number of spontaneous metastases produced by untreated hamster melanoma HMI-FS

cells (Scheicher et al., 1988).

A positive correlation has been shown between the in vitro recombinant basement membrane (RBM) invasion assay and in vivo metastasis (Albini et al., 1987; Repesh, 1989). The RBM model consists of a porous Transwell p.c. membrane (Costar Co., Cambridge, MA) that is coated with a reconstituted basement membrane (Matrigel, Collaborative Res., Bedford, MA). Matrigel is derived from the murine Engelbreth-Holm-Swarm (EHS) sarcoma cell line and consists of laminin, collagen type IV, heparan sulfate proteoglycan and entactin (Kleinman et al., 1986). During invasion, tumor cells locally degrade the Matrigel and migrate through the porous p.c. membrane. The cells can then be quantified on the underside of the p.c. membrane by light microscopy (Albini et al., 1987; Hendrix et al., 1989), radioactive labelling (Repesh, 1989) or scanning electron microscopy.

The RBM model has been used to test the effect of various drugs on the invasion process. Drugs tested include cytoskeletal agents such as Colchicine (Welch et al., 1989) and estramustine (Wang and Stearns, 1988). The two drugs chosen for this study, RA and BA, effect the cytoskeleton in some tumor cell lines (Ng et al., 1985; Lehtonen et al., 1983; Altenburg et al., 1976; Ryan and Higgins, 1988). I have previously reported the effect of these two agents on the migration of murine B16a cells (McGarvey and Persky, 1989. The short term effects of BA on the invasion process in the human amnion basement membrane (HABM) assay have been reported (Thorgeirsson et al., 1984). Pretreatment for 24 hrs with BA enhanced the invasion of M5076 sarcoma cells (Thorgeirsson et al., 1984). Likewise, pretreatment of murine

B16F10 melanoma cells for 24 hrs with RA increased invasion through the amnion. However, pretreatment with RA for 48 to 72 hrs decreased invasion (Wood et al., 1985)). Retinoic acid has been shown to inhibit invasion of tumor cells in the RBM assay without effecting cellular proliferation (Nakajima et al., 1989).

In my study, both in vitro and in vivo protocols were used to study invasion and metastasis. For the in vitro migration and invasion studies, B16a and K-1735 melanoma cells were treated with BA or RA for three to six days. For the in vivo study, syngeneic female C57BL6 mice were primed with all trans-RA or BA by subcutaneous implantation of drug pellets one day prior to tail vein injection of B16a cells.

MATERIALS AND METHODS

Cell Culture: Murine B16a and K-1735 melanoma cells were obtained from the DCT Tumor Repository, NCI-Frederick Cancer Research Facility (Frederick, MD). Murine NIH-3T3 cells were obtained from American Type Tissue Collection (Rockville, MD). Cells were cultured in Eagle's Minimum Medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and L-glutamine supplemented with sodium pyruvate (110 mg/ml) (Sigma, St. Louis, MO), 10% heat inactivated fetal bovine serum (FCS) (Gibco) or 10% NuSerum (Collaborative Res., Cambridge, MA), 1% penicillin G-streptomycin- amphotericin B (Fungizone) (Gibco), MEM non-essential amino acids (Gibco) (10 ml/l), sodium bicarbonate (1.27 g/l) (Sigma), and Hepes (5.96 g/l) (Sigma) (final pH 7.3). Cells were cultured in a humidified incubator (37^{o}C, 5% CO_2) and were refed every three days. Cells were subcultured with trypsin/EDTA (Sigma) during log phase growth.

B16a cells were also obtained from metastatic lung nodules or subcutaneous tumors according to the following protocol. The cells, originally obtained from the repository, were grown to preconfluence in T-75 flasks, trypsinized, and suspended in PBS without Ca^{++} and Mg^{++}. Four female C57BL6 mice received an injection of 0.2 ml of PBS-tumor cell suspension (500,000 cells/ml) either in a subcutaneous site or in the lateral tail vein. Mice were killed after 21 days. Subcutaneous or lung tumors were removed and minced in MEM containing FCS. After 24 hours of incubation, unattached cells and debris were removed from the tissue culture-treated dishes. The adherent subcutaneous or lung

nodule-derived cells were passaged twice in vitro and then used in the experiments without further passage.

Though a range of cytostatic concentrations of butyric acid (J.T. Baker, Phillipsburg, NJ) and retinoic acid (Sigma) was observed (McGarvey and Persky, 1989), a concentration of 1.5 mM BA and 1 X 10^{-6} M RA were used in all ^{3}H-IdUR incorporation, migration and invasion assays.

Incorporation of ^{3}H-Iododeoxyuridine: Untreated and BA-treated cells were incubated with 1 uCi/ml ^{3}H-Iododeoxyuridine (^{3}H-IdUR) (Amersham Inc., Arlington Hts., IL) for 24, 48 and 72 hrs. Butyric acid was simultaneously added with the ^{3}H-IdUR to the cells. Cells were lifted with trypsin/EDTA, filtered, and the bound radioactivity was determined as counts per minute (CPM) with a beta scintillation counter. The mean (n=4) and standard error of the mean (SE) were determined for each time point.

Adhesion Assay: Untreated and BA-treated B16a cells were labelled with 1 uCi/ml of ^{3}H-IdUR for 24 hrs. Labelled cells were plated onto uncoated or Matrigel-coated p.c. membranes and incubated for 72 hrs. The cell supensions above and below the membrane were collected individually following incubation with trypsin/EDTA and filtered. The membranes were collected, solubilized (Soluene 350, Packard Inc., Downers Grove, IL), and liquid scintillation fluor added. Counts per minute for each of the three fractions were determined by beta scintillation counting. The mean (n=8) and SE of each fraction were calculated. Students' t tests compared the means of the untreated BA-treated cells. An average of 0.05 counts per minute per cell were

plated onto the uncoated and Matrigel-coated membranes.

Key: The key for identification of the migration and invasion experiments follow an X-Y-Z format whereby X is the drug, Y is the treatment, and Z is the cell source. X may be C (control, i.e. untreated), BA or RA. Y may be PT (pretreatment), NP (no pretreatment) or NT (no treatment). Z may be LN (lung nodule derived), SC (subcutaneously-derived), EP (early passage, less than six in vitro passages), LP (late passage, greater than six in vitro passages), or K (K-1735 cells).

Migration Assay: The methodology was a modification of the migration assay of Varani et al. (1978). Our modifications of this procedure have been reported in detail (McGarvey and Perksy, 1989).

Three different migration experiments were performed on B16a cells (Figure #2). Experiment #1 compared the migration of untreated lung nodule-derived (C-NT-LN), untreated subcutaneously-derived (C-NT-SC) and BA-pretreated lung nodule derived (BA-PT-LN) B16a cells. Experiment #2 compared the migration of untreated (C-NT-LN), RA-treated (RA-NP-LN), and RA-pretreated (RA-PT-LN) lung nodule-derived cells. Experiment #3 compared the migration of untreated subcutaneously-derived (C-NT-SC) and RA-pretreated subcutaneously-derived (RA-PT-SC) cells. Each experiment was done at least in triplicate. In each experiment, 5×10^4 cells in appropriate media (MEM+FCS with or without BA or RA) were plated in the Transwell insert (inner well). The matching outer wells similarly contained MEM + FCS with or without BA or RA, but no cells. Drug pretreatment indicated that the cells were incubated in RA or BA for 72 hrs before the migration assay in T75

flasks, and for a further 72 hr incubation period during the migration assay. Non-pre-treated cells were incubated with BA or RA only during the time of the migration assay. At the conclusion of the migration assay, membranes were processed for SEM. Cells that migrated to the bottom of the membrane were quantified by counting cells in 50 random fields at 500X. A Students' t test was performed on the results of experiment 3. For all other migration experiments, Welch's tw (t') unequal variance or Bechhofer-Dunnett-Krishnaiah-Armitage equal variance multiple comparison tests were performed (Wilcox, 1987).

Invasion Assay: The invasion assay consisted of a Transwell chamber with an 8 um diameter pore p.c. membrane coated with Matrigel. Matrigel was diluted 1:20 (71 ug of protein/well) and added to Transwell inserts kept on ice (Repesh, 1989). After overnight dehydration, Matrigel was rehydrated for two hrs with serum free MEM. The MEM was removed, and cells were added to the top wells of the Transwell insert (50,000 cells/well). In some experiments, cells were preincubated with RA or BA for 72 hr prior to being plated on the Matrigel. At the time of plating, RA or BA was added to the top and bottom wells. Two sets of controls were done, one containing medium alone the other containing medium with 0.1% ethanol (the carrier for RA). After 72 hrs, membranes were processed for SEM.

Invasive cells were quantified as described in the migration assay with the noticeable exception that for selected experiments, the entire bottom surface of the membrane was scored for invasive cells rather than 50 fields at 500 X. Each experiment was performed at least in triplicate. Six different invasion experiments were done (see Fig. 3).

Experiment #1 compared the invasion rates of untreated early passage (< 6 passages) (C-NT-EP), untreated late passage (>6 passages) (C-NT-LP), BA-treated late passage (BA-NP-LP), and BA-pretreated late passage (BA-PT-LP) B16a cells. Experiment #2 compared the invasion rates of untreated (C-NT-SC), BA-treated (BA-NP-SC) and BA-pretreated (BA-PT-SC) subcutaneously-derived B16a cells. Experiment #3 compared the invasion rates of untreated subcutaneously-derived (C-NT-SC) (plated at 50,000 or 25,000 cells/well), untreated lung nodule-derived (C-NT-LN) (plated at 50,000 cells/well), BA-treated lung nodule-derived (BA-NP-LN) and BA-pretreated lung nodule derived (BA-PT-LN) B16a cells (plated at 50,000 cells/ml). Experiment #4 compared the invasion rates of untreated lung nodule-derived (C-NT-LN), RA-treated lung nodule-derived (RA-NP-LN) and RA-pretreated lung nodule-derived (RA-PT-LN) B16a cells. Experiment #5 compared the invasion rates of untreated (C-NT-K) and RA-pretreated (RA-PT-K) K-1735 cells. Experiment #6 compared the invasion rates of untreated (C-NT-LN) and BA-pretreated (BA-PT-LN) lung nodule derived cells in the presence of NIH-3T3 cell conditioned medium for 24 and 48 hrs. The conditioned medium was obtained by incubating preconfluent NIH-3T3 cells for 24 hrs in serum-free medium containing 1% bovine serum albumin and 0.005% ascorbic acid (Albini et al., 1986). This conditioned medium was added to the outer well. After 24 hrs, cells were refed with fresh media with or without BA. Fresh conditioned medium was also added to the outer well. A students' t test was performed on the results of experiment five. For all other invasion experiments, Welch's tw (t') unequal variance or Bechhofer-Dunnett-Krishnaiah -Armitage equal variance multiple

comparison tests were performed.

The In Vivo Assay: The day before tumor cell injection via lateral tail vein methodology, each mouse was inplanted subcutaneously with a pellet containing 5 mg of all trans-RA, BA, or placebo (Innovative Res., Toledo, OH). Pellets release the drugs by zero order kinetics. A 5 mg pellet over 20 days releases 250 ug/day (10 ug/hr) of RA or BA. In addition, a steady state for each drug is reached after two to four hours (S. Shafie, personal communication). Lung nodule-derived and subcutaneous-derived B16a cells were suspended in PBS in a 1:1 ratio. The concentration of cells was adjusted to 500,000 cell/ml. Cell viability before tail vein injections was always greater than 90% as determined by the trypan blue exclusion method. A 0.2 ml cell suspension was injected into the lateral tail vein of 46 day old female C57BL6 mice (Harlem Sprague Dawley, Indianapolis, IN). The mice were divided into 25 controls and 50 experimental. Fifteen (5 control, 5 RA-treated, and 5 BA-treated) mice received tail vein injections per day in order to maximize the proficiency of the injection protocol. Cell viability was also determined to be greater than 90% in the cell suspension not injected into the mice. Thus the subcutaneous pellet implantation and tail vein injection protocols were spread over five days. Mice were killed after 21 days. Lungs were fixed in Bouin's fixative and the metastatic nodules were counted with a stereo dissecting microscope at 6.4 X. Gross examination of the thoracic-abdominal viscera and body walls was performed. Mann-Whitney U nonparametric test was performed to determine statistical significance (Wilcox, 1987).

RESULTS

Butyric acid reduced the incorporation of ^{3}H-IdUR in B16a cells within the first 24 hrs of drug incubation. The percent inhibition of ^{3}H-IdUR incorporation by BA as compared to the control was 79%, 64% and 71% at 24, 48 and 72 hrs, respectively (Fig. 1). There is a dramatic increase in cpm from 24 to 48 hrs followed by a leveling off thereafter.

Adhesion Assay: Butyric acid increased significantly the adhesion of ^{3}H-IdUR labelled B16a cells, which were incubated for 72 hr, to both uncoated and Matrigel-coated p.c. membranes (45%, $p<.01$; 66%, $p<.0001$, respectively). After fifteen minutes of incubation in a trypsin/EDTA solution, 43.3% ($\pm$6.3%) and 76.8% ($\pm$2.9%) of BA-treated cells were still bound to the uncoated and Matrigel-coated membranes, respectively, as compared to 23.9% ($\pm$3.0%) and 30.2% ($\pm$4.6%) for untreated cells.

Migration Assay: Figure 2 illustrates the mean $\pm$ SE of the three migration experiments. For each experiment, percent migration of the untreated (control) cells was adjusted to 100%. Percent migration is defined as the number of cells plated on top of the p.c. membrane divided by the number of cells calculated to be on the bottom membrane surface multiplied by 100.

In the first experiment, 1.08$\pm$0.18 lung nodule-derived untreated cells were counted per field on the bottom of the p.c. membrane (percent migration 1.7%). In contrast, 4.35$\pm$0.79 subcutaneously-derived untreated cells were counted per field (percent migration

6.7%). Subcutaneously-derived untreated cells had an increased migration rate (403%, $p<0.05$) compared to lung nodule-derived untreated cells. The migration rate of lung nodule-derived cells was increased by pretreatment with BA (667%, $p<0.05$). The number of BA-pretreated cells counted per field was 7.21 ± 0.86 (percent migration was 11.1%). If the migration rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 1), BA-pretreatment further enhanced migration (2900% increase, $p<0.05$).

In the second experiment, 1.13 ± 0.25 lung nodule-derived untreated cells were counted per field (percent migration 1.7%). RA-treatment (0.59 ± 0.14; percent migration 0.9%) or RA-pretreatment (2.24 ± 0.48; percent migration 3.4%) did not significantly effect the migration rate of lung-nodule derived cells. The percent migration values of untreated lung nodule-derived cells were similiar for experiments 1 and 2. If the migration rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 2), RA-treatment and RA-pretreatment enhanced migration (246% increase, $p<0.05$; 934% increase, $p<0.05$, respectively).

In contrast to the migration data of lung nodule-derived cells of Exp. 2, RA-pretreatment of subcutaneously-derived cells did decrease migration (62% decrease, $p<0.001$) (Exp. 3). For the subcutaneously-derived cells, 4.48 ± 0.38 untreated cells (percent migration 6.7%) and 1.67 ± 0.22 RA-pretreated cells (percent migration 2.6%) were counted per field. If the migration rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 2), then RA-pretreatment did not significantly enhance

migration. The rationale for using or not using a proliferation factor to determine migration rates is found in the Discussion. It is noteworthy that the percent migration values of subcutaneously-derived untreated cells for Exps. 1 and 3 were similiar.

Invasion Assay: For each of the six invasion experiments, the percent invasion of untreated cells was adjusted to 100% (Fig. 3). The untreated cells were derived from three different sources, i.e. from stock received from the tumor depository (Exps. 1 and 5), from subcutaneous passage (Exp. 2), and from tail vein passage (Exps. 3,4,6). The invasion rates were dependant on the derivation of the cells.

Invasion experiments 1-3 demonstrate that (1) BA increases invasion and (2) lung nodule derived cells are more invasive than subcutaneously derived cells.

In the first invasion experiment, there was no significant difference amoung the invasion of untreated late passage (0.13 ± 0.12 cells, percent invasion 0.20%), untreated early passage (0.22 ± 0.02 cells per field, percent invasion 0.34%), and BA-treated late passage (0.29 ± 0.05 cells, percent invasion 0.45%) B16a cells. There was, however, a significant increase in the invasion rate (490%) of BA-pretreated late passage cells as compared to both early and late passage untreated cells ($p<0.05$, $p<0.05$, respectively). An average of 0.64 ± 0.10 BA-pretreated late passage cells were counted per field (percent invasion 0.98%). If the invasion rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 1), BA-treatment and BA-pretreatment further enhanced invasion

(940% increase, $p<0.05$; 2100% increase, $p<0.05$).

In the second experiment, the invasion rate of BA-treated and BA-pretreated subcutaneously-derived cells was significantly increased ($p<0.05$ and $p<0.05$, respectively) as compared to untreated subcutaneously-derived cells. Since there were few untreated subcutaneous-derived cells on the bottom of the membrane, cells on the entire bottom surface of the membrane were counted. An average of 30.7 untreated cells were counted per membrane; percent invasion 0.12%. On the other hand, an average of 1.81 ± 0.37 BA-treated and 10.15 ± 0.60 BA-pretreated cells were counted per field. Percent invasion for BA-treated and BA-pretreated cells was 2.8% and 15.4%, respectively. If the invasion rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 1), BA-treatment and BA-pretreatment further enhanced invasion (17600% increase, $p<0.05$; 110000% increase, $p<0.05$).

In the third invasion experiment, there was no significant difference in the invasion rate of subcutaneously-derived untreated cells plated at either 25,000 cells or 50,000 cells/well. An average of only 4.7 and 5.7 cells were counted on each of the triplicate membranes. Percent invasion for 25,000 and 50,000 cells was 0.02% and 0.04%, respectively. All experiments except this one plated 50,000 cells/well. There was, however, a significant increase in the invasion of lung nodule-derived untreated cells ($p<0.05$, 0.73 ± 0.10 cells per field, percent invasion 1.1%). Butyric acid treatment or pretreatment increased the invasion rate of lung nodule-derived cells (238%, $p<0.05$ and 673%, $p<0.05$, respectively). An average of 1.74 ± 0.36 BA-treated

cells (percent invasion 2.67%) and an average of 4.91±0.02 BA-pretreated cells (percent invasion 7.5%) were counted per field. If the invasion rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 1), BA-treatment and BA-pretreatment further enhanced invasion (1051% increase, $p<0.05$; 2936% increase, $p<0.05$, respectively).

Experiments four and five illustrate the effect of RA on two cell lines that have different invasive potential. The percent invasion of untreated B16a cells (Fig. 3, Exp. 4) and of untreated K-1735 cells (Fig. 3, Exp. 5) was 0.13% and 23.6%, respectively. For Exp. 4, there was an average of 33.6 untreated, 15.2 RA-treated, 43.8 RA-pretreated cells counted per membrane. The percent invasion was 0.13%, 0.06%, and 0.18%, respectively. Thus, invasion was inhibited 54% by RA-treatment ($p<0.05$), but not by RA-pretreatment. If the invasion rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 2), RA-treatment and RA-pretreatment enhanced invasion (295% increase, $p<0.05$; 853% increase, $p<0.05$). For Exp. 5, an average of 1.33±0.38 untreated and 0.38±0.07 RA-pretreated K-1735 cells were counted per field at 1700X (instead of the usual 500X). This higher magnification was selected to properly deliniate cell boundaries due to the large number of invasive cells. The percent invasion per 50,000 cells plated was 23.6% and 6.7%, respectively. There was a 72% decrease in invasion of RA-pretreated cells ($p<0.001$). If the invasion rates were formulated to include cellular proliferation during the 72 hr incubation (Chapter IV, Fig. 2), RA-pretreatment enhanced invasion (132% increase, $p<0.05$).

The sixth experiment determined the affect of conditioned medium on lung nodule-derived B16a cell invasion. Conditioned medium was placed in the bottom well and the invasion assay was reduced from the standaand 2.02±0.29 BA-pretreated cells were counted per field. Percent invasion was 0.60% and 3.10%, respectively. For 48 hrs, 1.70±0.09 untreated and 6.15±0.29 BA-pretreated cells were counted per field. Percent invasion was 2.6% and 9.4%, repectively. Thus, experiment #6 has two key points. First, conditioned medium enhanced invasion. The percent invasion of untreated lung nodule derived cells after 48 hrs in the conditioned medium (Fig. 3, Exp. 6) was 233% greater than for lung nodule-derived cells after 72 hrs without conditioned medium (Fig. 3, Exp. 3). Second, BA-pretreatment significantly increased invasion in 24 hrs by 518% and in 48 hrs by 362% as compared to untreated cells of the same incubation period.

In Vivo Assay: Both RA or BA, which were administered by subcutaneous pellet, reduced experimental metastasis ($p<0.05$ and $p<0.05$, respectively) according to the Mann-Whitney U test (Fig. 4). Butyric acid decreased the number of lung nodules as compared to the placebo pellet by 91.4% (mean decrease) or 99% (median decrease). Retinoic acid decreased the number of lung nodules as compared to the placebo pellet by 80% (mean decrease) or 84% (median decrease). Furthermore, two mice were completely tumor-free in the RA-treated group and nine mice were tumor-free in the BA-treated group. Gross examination of the thoracic-abdominal viscera and body walls revealed that all metastatic foci were confined to the lungs. Figure 6 illustrates lungs from untreated, RA, and BA-treated animals.

DISCUSSION

The experimental metastatic assay contains different steps associated with the formation of lung tumors (Fidler, 1970). These include blood borne transport, extravasation and tumor growth. One or more steps of the assay can be effected by BA and RA. I used ^{3}H-IdUR incorporation, adhesion, migration and reconstituted basement membrane (RBM) assays as reductionist tools to understand the metastatic process.

I have shown that 1 X 10^{-6} M RA and 1.5 mM BA produce cytostasis without cytotoxicity in the B16a cell line (McGarvey and Persky, 1989). I have also emphasized the importance of using the cytostatic concentration of potential anti-invasive drugs in in vitro studies (McGarvey and Persky, 1989). Retinoids, which include all trans-retinoic acid, induce tumor cell differentiation, inhibit tumor cell growth (G1 phase of the cell cycle), suppress tumor cell phenotypic properties, and stimulate the host immune response (for a review see Roberts and Sporn, 1984). Butyric acid similiarly induces differentiation and inhibits proliferation (G1 phase of the cell cycle) of tumor and nontumor cells (Kruh, 1982).

In my study, BA decreased ^{3}H-IdUR incorporation within the first 24 hours of incubation. Despite this reduction in proliferation, the invasion rate increased with longer exposure to BA (Fig. 3, Exps. 1-3). Drug concentrations necessary to inhibit in vitro invasion may be similiar or lower than that needed to inhibit proliferation in vitro. For example, RA reduced invasion of rat mammary adenocarcinoma 13762NF

cells in vitro without inhibiting proliferation (Nakajima et al., 1989).

A shortened time period for the invasion assay would be advantageous to distinguish drug induced inhibition of proliferation from drug induced inhibition of invasion. Therefore, I used conditioned medium to increase invasion. There was an increase in invasion of untreated and BA-pretreated lung nodule-derived cells in 48 hrs using conditioned medium (Fig. 3, Exp. #6) as compared to 72 hrs without conditioned medium (Fig. 3, Exp. #3). A further enhancement of the migration and invasion rate by BA was observed if the 72 hr proliferation data (Fig. 1, Chapter IV) were included in determining these rates (Table 1 and 2).

Retinoic acid decreased the migration and invasion rates of Bl6a and Kl735 cells. These findings support the experiments of Nakajima et al. (1989). However, if a proliferation factor was used to calculate migration and invasion rates, then RA enhanced both migration and invasion. The usefulness of a proliferation factor in calculating migration and invasion rates can be questioned in light of my results obtained from calf serum treated cells. For example, the migration of RA-treated Bl6a cells was significantly decreased compared to untreated and calf serum treated Bl6a cells. Both RA and calf serum significantly decreased proliferation as compared to untreated cells (McGarvey and Persky, 1989). The concern of using a factor to "correct" for cellular proliferation in the migration experiments is also applicable to the invasion experiments. Therefore, whether RA decreases or increases migration or invasion of murine melanoma cells

would be answered by shortening the duration of the assay so that proliferation would not be a factor.

In this in vivo study, it is possible that once the tumor cells invaded the lung paranchyma the growth of the lung colonies was inhibited by the continuous exposure to BA or RA. This would enhance the ability of host defenses to eradicate or reduce the tumors. Retinoids administered by i.p. injection or orally have been shown to reduce the size of xenotransplanted subcutaneous human mammary carcinoma (MDA-MB-231) (Halter et al., 1988)), murine embryonal carcinoma (McCue et al., 1988) and murine B16F10 melanoma (Drewa and Schachtschabel, 1985). However, the precise role of growth inhibition in experimental metastasis is unknown. Only recently has inhibition of primary tumor growth been separated from the reduction of spontaneous metastasis by retinoids in a hamster melanoma-athymic mouse model (Scheicher et al., 1988).

Tumor cell adhesion in vitro is important in theories of cellular invasion (Liotta et al., 1983). I have shown that BA pretreatment increases heterotypic adhesion between B16a cells and p.c. membranes as well as Matrigel coated p.c. membranes. This increase in adhesion correlates with increased in vitro invasion of BA-treated B16a cells. In contrast to the increased cellular adhesion to Matrigel induced by BA, a decrease in the number of laminin receptors has been reported in the BA-treated human pancreatic carcinoma cell line (PANC-1) (Bryant et al., 1986). Matrigel contains collagen type IV, heparin sulfate proteoglycans and entactin as well as laminin. Therefore, it is possible that a decrease in binding to one element of a basement

membrane does not imply a decrease in binding to all components.

Tumor cell adhesion in vivo is central to the 'seed and soil' hypothesis of Paget (1889). Decreased adhesion has been correlated with both increased and decreased experimental metastasis (Takanaga, 1984; Takanaga, 1986; Raz and Ben-Ze'ev, 1983; Werling et al., 1986). Butyric acid has been shown to increase homotypic and heterotypic adhesion of Lewis lung carcinoma (P-29) cells and pretreated cells have been shown to have increased experimental metastasis (Takanaga, 1986).

In contrast to the increase in heterotypic adhesion induced by BA, RA has been shown to decrease the adhesion of a B16F1 variant to elements of the basement membrane (laminin, entactin and collagen type IV) and collagenous stroma (collagen type I) (Edward et al., 1989). This decrease in adhesion may be one of the factors that decreased in vitro invasion and experimental metastasis in my study. The same reasoning may apply to the results observed in in vitro invasion assays (Fazely et al., 1988; Fazely et al., 1985; Nakajima et al., 1989) and experimental metastasis assays (Edward et al., 1989; Couch et al., 1987).

The role of proteolytic enzymes in the degradation of the extracellular matrix by tumor cells in invasion has been correlated with the in vivo metastatic potential of many tumor cell lines including the B16 melanoma cell line (Wang et al., 1980; Sloane et al., 1982; Liotta et al., 1980). Both BA and RA have been shown to modulate the activity and production of degradative enzymes. Increased invasion of M5076 sarcoma cells in the HABM assay by butyric acid has been correlated with increased type IV collagenase activity (Thorgeirsson et

al., 1984). The increase in collagenase type IV activity supports my observation that BA-treatment enhances invasion. On the other hand, RA has been shown to decrease the degradation of the collageneous capsule surrounding subcutaneous murine tumors (Takahashi et al., 1985), and to decrease both type IV collagenase and the invasion of 13762NF cells in the RBM assay (Nakajima et al., 1989). The reported decrease in type IV collagenase by RA-treatment likewise supports my finding that RA treatment decreased the invasion of B16a and K-1735 cells in the Matrigel assay.

There appears to be a correlation between increased migration and increased invasion. The K-1735 cells used in this study are highly invasive and highly migratory (Silberman et al., 1990). There are, however, exceptions to the theory that highly migratory cells are highly invasive. For example, subcutaneously-derived cells were significantly more migratory than lung nodule-derived cells. This may be due to the heterogeneous nature of the subcutaneously-derived cells. On the other hand, lung nodule-derived cells invaded the Matrigel in significantly greater numbers than subcutaneously-derived cells. This is expected since the lung nodule-derived cells represent a special subpopulation that are invasive in vivo. Selection for invasive or migratory cells has already been reported. For example, repeated passage of invasive cells in the RBM assay produced more invasive cells (Wang and Stearns, 1988; Hendrix et al., 1989). Likewise, cells obtained from repeated serial passages through the human amnion have produced more experimental metastases (Persky and Chmielewski, 1987) and spontaneous metastases (Tullberg et al., 1989). In contrast,

hypermotile cells from repeated passage through microporous membranes decreased the number of experimental and spontaneous metastases (Grimstad, 1988). It is possible that selection of either the migratory or the invasive phenotype may select a subpopulation that is not as competent at completing another step of the metastatic cascade.

The cells used in the in vitro assays were derived from multiple sources (the tumor depository, subcutaneous tumors and lung nodules) and had different migration and invasion rates. Therefore, each migration and invasion experiment must be independently evaluated. The use of cells from different sources, different passage numbers or different cell lines should not cloud the main in vitro concept that BA increases migration and invasion and that RA decreases migration and invasion. An important observation in the migration and invasion assays is that retinoic acid has a greater effect on highly invasive (K-1735 cells) or highly migratory cells (subcutaneously derived B16a cells) whereas butyric acid has a greater effect on the migration and invasion of low invasive cells (McGarvey and Persky, 1989). However, if the decrease in proliferation induced by RA and BA is included in the invasion rates, RA and BA enhanced invasion. In addition, increasing the in vitro invasion rate by either using lung nodule-derived cells or conditioned medium did not obliterate the overall effect of BA on increasing in vitro invasion.

I believe that drug administration by pellet prior to tumor cell injection primed the animal. Administration by subcutaneous pellet allowed for constant drug exposure during the entire experiment, unlike i.p., i.v. or dietary intake. Blood serum levels of mice that received

a 5 mg pellet of RA have been shown to contain 5 X 10^{-8} M RA (Mariotti et al., 1987). Tissue concentrations of RA were not reported in this study. However, administration of all trans-RA by intravenous injection found that tissue concentrations were higher than blood serum levels (Wang et al., 1980). Pellets containing higher concentrations resulted in toxicity to the mice (Mariotti et al., 1987). I have not determined the murine serum levels of BA in this study. However, the animals in these experiments did not show signs of BA toxicity. The BA concentration was based on information provided by Dr. S. Shafie from Innovative Research Inc. (personal communication). To my knowledge, no information is available on blood serum or tissue concentrations of BA by pellet administration.

Although BA and RA have a myriad of effects on the multiple steps of the metastatic cascade, both drugs significantly reduced in vivo experimental metastasis in our study. Further combined in vitro and in vivo investigations with these drugs and other differentiation agents are warranted.

Figure 1: Counts per minute (CPM) of ^{3}H-IdUR labelled cells plotted at 24, 48, and 72 hrs. as the mean (n=4) $\pm$ standard error of the mean.

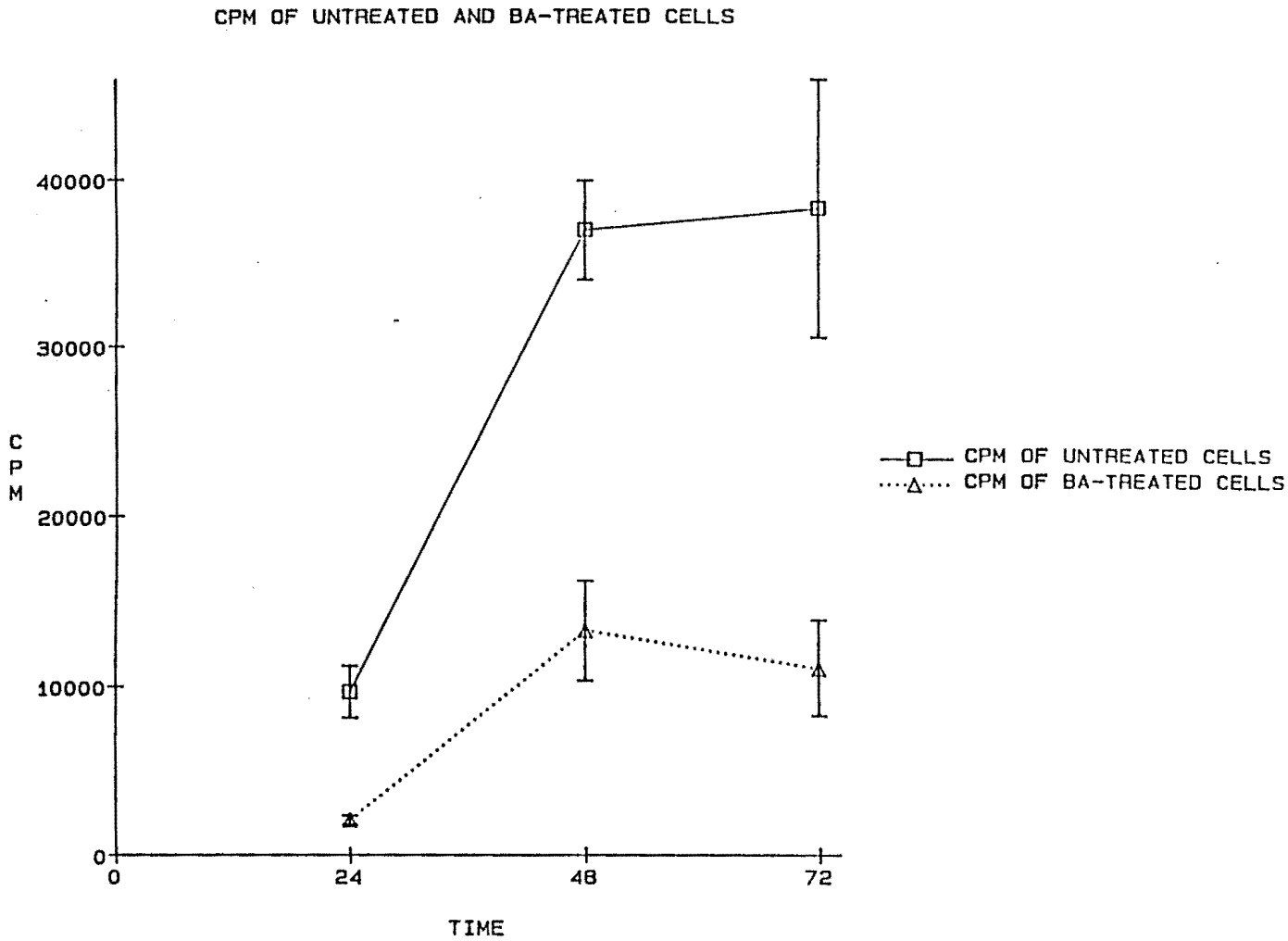
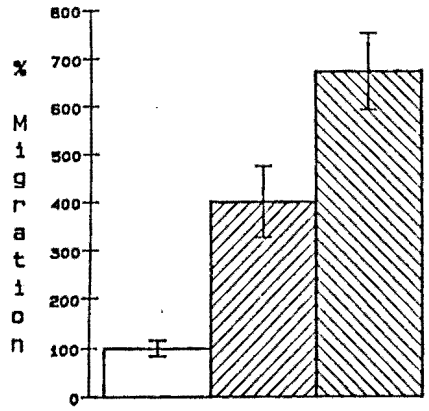
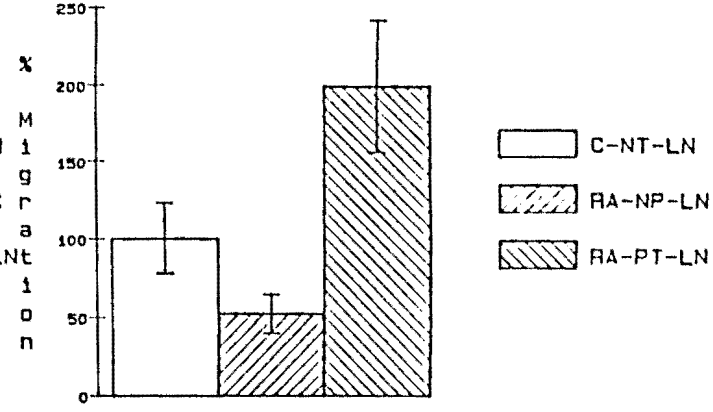

CPM OF UNTREATED AND BA-TREATED CELLS
40000
30000
20000
10000
0
C
P
M
0
24
48
72
TIME
CPM OF UNTREATED CELLS
CPM OF BA-TREATED CELLS

Figure 2: The percent migration of B16a cells. Three separate experiments were done. For details see "Migration Assay" in the results section. Each bar is the percent migration $\pm$ standard error of the mean. An asterisk (*) represents a value that is significantly different from the controls.

MIGRATION EXPERIMENT #1



MIGRATION EXPERIMENT #2



MIGRATION EXPERIMENT #3

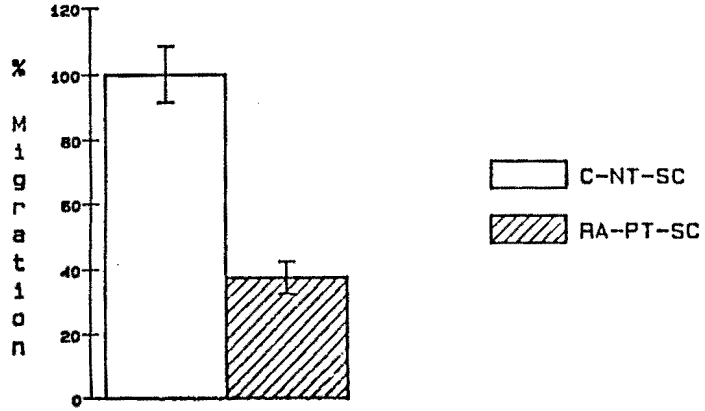
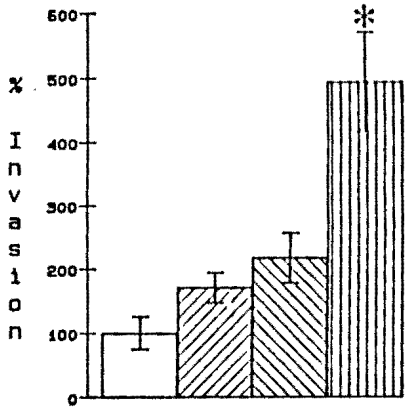
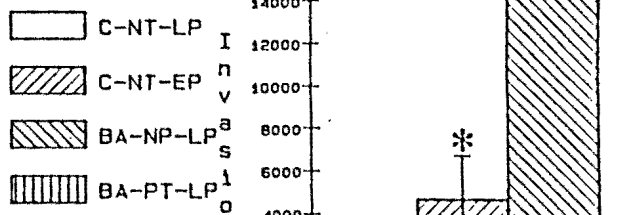


Figure 3: The percent invasion of B16a cells. Four different experiments were performed. For details see "Invasion Assay" in the results section. Each bar is the percent invasion $\pm$ standard error of the mean. An asterisk (*) represents a value that is significantly different from the controls. In experiment #3, the percent invasion of lung nodule-treated cells was significantly different from the percent invasion of the subcutaneously-derived cells plated at either 25,000 or 50,000 cells/well.

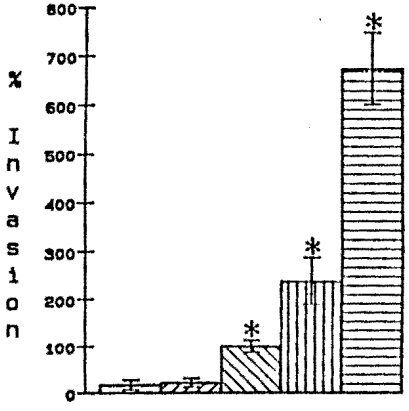
INVASION EXPERIMENT #1

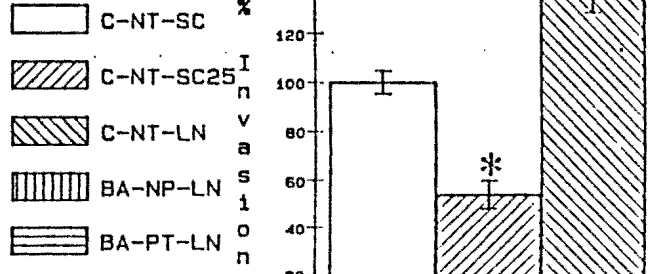




INVASION EXPERIMENT #2

INVASION EXPERIMENT #3

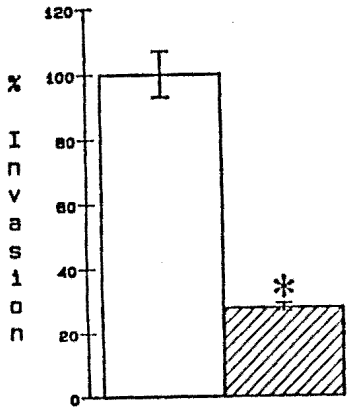




INVASION EXPERIMENT #4

Figure 4: The percent invasion of B16a and K-1735 cells. Two differrent experiments were performed. For details see "Invasion Assay" in the results section. Each bar is the percent invasion $\pm$ standard error of the mean. An asterisk (*) represents a value that is significantly different from the controls. The double asterisk (**) in experiment #6 represents a value that is significantly different from the controls at 24 hrs.

INVASION EXPERIMENT #5



INVASION EXPERIMENT #6

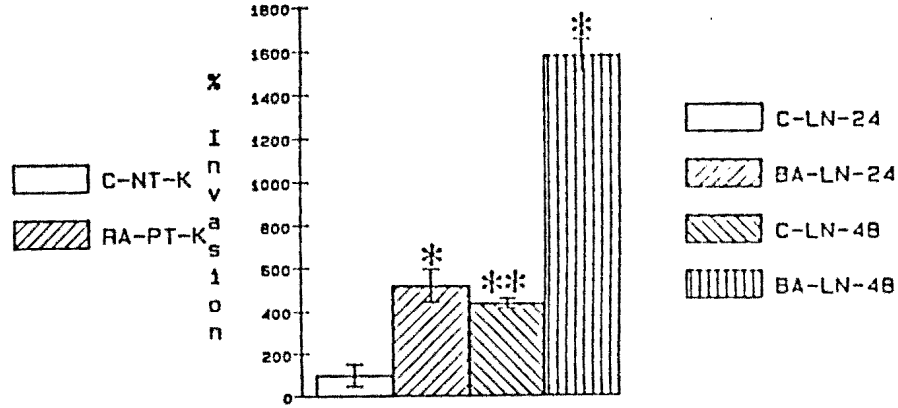


Figure 5: The in vivo experimental data. Subcutaneous pellets containing either a placebo, BA, or RA were implanted into C57BL6 mice one day prior to injection via tail vein with B16a cells. Mice were killed 21 days after tumor cell injection. An average of 102.7±49.4 (mean ± S.D.), 8.8±14.4, and 20.3±15.8 lung nodules were counted per the placebo, BA, and RA-treated groups, respectively. The median scores were also determined for each group.

IN VIVO EXPERIMENTAL METASTASIS

CELL LINE	TREATMENT (# of mice)	# OF SURFACE LUNG METASTASES	MEDIAN	MEAN	S.D.
Murine B16a Melanoma	Placebo (19)	1, 20, 48, 58, 59, 75, 81, 88, 105, 124, 125, 128, 133, 133, 137, 139, 150, 162, 185	124	102.7	49.4
	Butyric Acid (21)	0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 1, 1, 2, 4,4, 14, 22, 22, 24, 41, 48	1	8.8	14.4
	All Trans-retinoic Acid (23)	0, 0, 1, 2, 3, 3, 7, 8, 11, 14, 18, 20, 22, 26, 29, 29, 31, 35, 38 39, 41, 44, 47	20	20.3	15.8

Figure 6. A photograph of lungs from control (placebo) treated animals. The lungs in Figs. 6, 7 and 8 were printed at different magnifications.

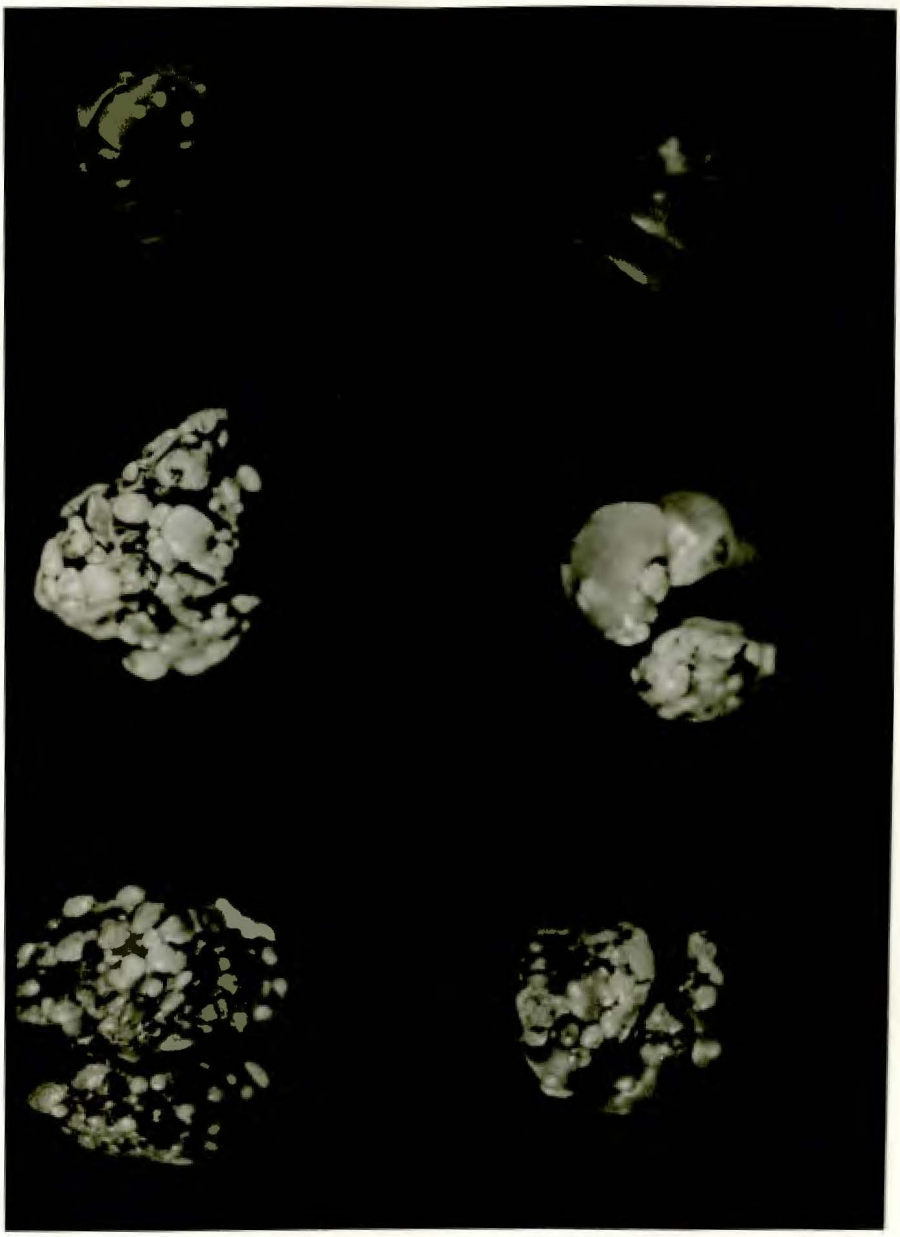


Figure 7. A photograph of lungs from BA-treated animals.

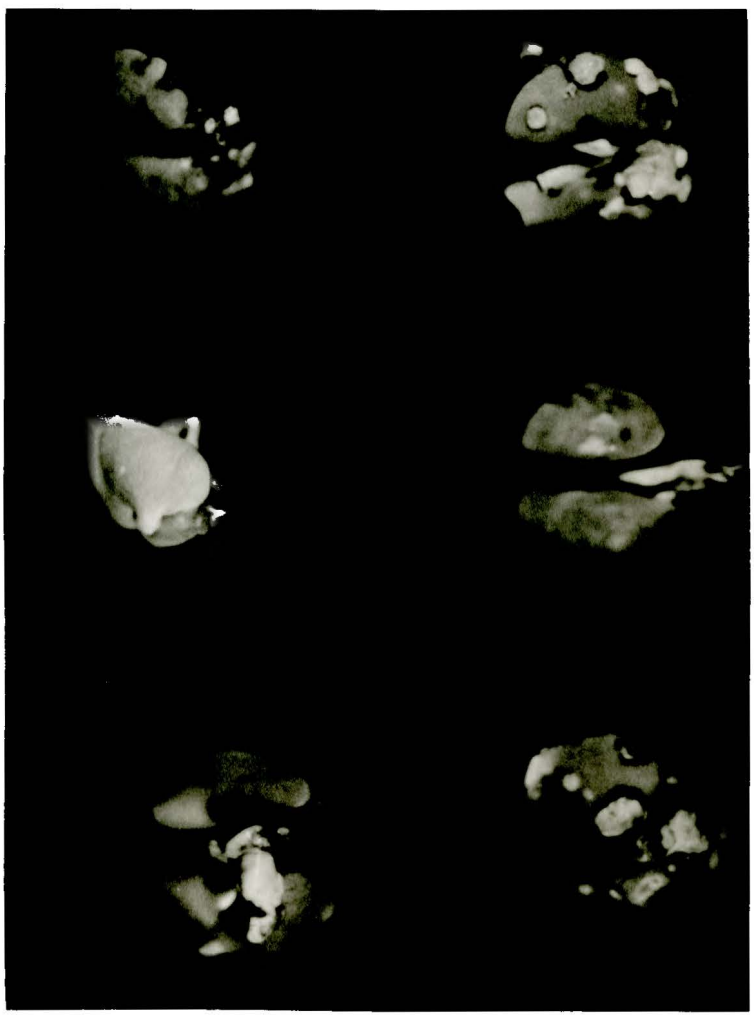


Figure 8. A photograph of lungs from RA-treated animals.

TABLE 1.

The migration rate of untreated, BA-treated, BA-pretreated, RA-treated and RA-pretreated cells after 72 hr incubation with a proliferation factor incorporated into the migration rate. The migration value was defined as the number of the cells per field times 50 divided by the final hemacytometer cell population (Fig. 1 and Fig. 2, Chapter IV). There was a significant increase in migration of BA-treated and BA-pretreated cells as compared to untreated B16a cells ($p<0.05$, $p<0.05$, respectively). There was a significant increase in migration of lung nodule-derived RA-treated and RA-pretreated cells as compared to untreated B16a cells ($p<0.05$, $p<0.05$, respectively).

MIGRATION VALUES

(INCLUDING THE PROLIFERATION RATE)

MIGRATION EXPERIMENT 1

	LUNG NODULE CELLS	SUBCUTANEOUS CELLS	PRE-BUTYRATE
N	4	4	3
MEAN	6.93×10^{-5}	28.0×10^{-5}	203×10^{-5}
SD	1.62×10^{-5}	7.30×10^{-5}	42×10^{-5}
SEM	3.60×10^{-5}	3.60×10^{-5}	24×10^{-5}
% MIG	---	404*	2929*

MIGRATION EXPERIMENT 2

	LUNG NODULE CELLS	RETINOIC ACID	PRE-RETINOIC ACID
N	3	3	6
MEAN	0.72×10^{-4}	1.77×10^{-4}	6.73×10^{-4}
SD	0.28×10^{-4}	0.72×10^{-4}	3.53×10^{-4}
SEM	0.16×10^{-4}	0.41×10^{-4}	1.44×10^{-4}
% MIG	---	246*	934*

MIGRATION EXPERIMENT 3

	SUBCUTANEOUS CELLS	PRE-RETINOIC ACID
N	3	6
MEAN	2.86×10^{-4}	5.01×10^{-4}
SD	0.44×10^{-4}	1.67×10^{-4}
SEM	0.25×10^{-4}	0.68×10^{-4}
% MIG	---	175

* - indicates a significant difference at $p < 0.05$.

TABLE 2.

The invasion rate of untreated, BA-treated and BA-pretreated cells after 72 hr incubation with a proliferation factor incorporated into the invasion rate. The invasion value was defined as the number of the cells per field times 50 divided by the final hemacytometer cell population (Fig. 1, Chapter IV). There was a significant increase in invasion of BA-treated and BA-pretreated cells as compared to untreated B16a cells ($p<0.05$, $p<0.05$, respectively).

INVASION VALUES

(INCLUDING THE PROLIFERATION RATE)

N	5	6	5
MEAN	0.86×10^{-5}	8.07×10^{-5}	17.9×10^{-5}
SD	0.49×10^{-5}	3.56×10^{-5}	6.30×10^{-5}
SEM	0.22×10^{-5}	1.45×10^{-5}	2.80×10^{-5}
% INV	___	938*	2081*

INVASION EXPERIMENT 2

	SUBCUTANEOUS CELLS	BUTYRATE	PRE-BUTYRATE
N	4	4	4
MEAN	3.98×10^{-5}	700×10^{-5}	4350×10^{-5}
SD	1.09×10^{-5}	330×10^{-5}	320×10^{-5}
SEM	0.54×10^{-5}	165×10^{-5}	160×10^{-5}
%INV	---	17588*	109296*

INVASION EXPERIMENT 3

	LUNG NODULE CELLS	BUTYRATE	PRE-BUTYRATE
N	4	4	4
MEAN	4.70×10^{-5}	49.4×10^{-5}	138×10^{-5}
SD	1.26×10^{-5}	14.5×10^{-5}	21.1×10^{-5}
SEM	0.63×10^{-5}	7.20×10^{-5}	10.5×10^{-5}
%INV	---	1051*	2936*

* - indicates a significant difference at $p < 0.05$.

TABLE 3.

The invasion rate of untreated, RA-treated and RA-pretreated cells after 72 hr incubation with a proliferation factor incorporated into the invasion rate. The invasion value was defined as the number of the cells per field times 50 divided by the final hemacytometer cell population (Fig. 2, Chapter IV). There was a significant increase in invasion of lung nodule-derived RA-treated and RA-pretreated cells as compared to untreated B16a cells ($p<0.05$, $p<0.05$, respectively). There was a significant increase in invasion of RA-pretreated K1735 cells as compared to untreated K1735 cells ($p<0.05$).

INVASION VALUES

(INCLUDING THE PROLIFERATION RATE)

INVASION EXPERIMENT 4

	LUNG NODULE CELLS	RETINOIC ACID	PRE-RETINOIC ACID
N	5	6	4
MEAN	3.1×10^{-5}	9.0×10^{-5}	26.1×10^{-5}
SD	0.81×10^{-5}	2.4×10^{-5}	8.0×10^{-5}
SEM	0.08×10^{-5}	1.0×10^{-5}	4.0×10^{-5}
%INV	---	295*	853*

INVASION EXPERIMENT 5

	UNTREATED - K1735 CELLS	PRA - K1735 CELLS
N	4	4
MEAN	34×10^{-4}	45×10^{-4}
SD	1.6×10^{-4}	3.0×10^{-4}
SEM	0.3×10^{-4}	1.0×10^{-4}
% INV	---	132*

* - indicates a significant difference at $p < 0.05$.

CHAPTER VI

QUANTIFICATION OF F-ACTIN IN MIGRATORY AND INVASIVE MURINE B16a MELANOMA CELLS

ABSTRACT

Butyric acid (BA) and retinoic acid (RA) affect the cytoskeleton (McGarvey and Persky, 1989). In this study, F-actin of migratory, nonmigratory, invasive and noninvasive murine B16a melanoma cells was digitized and quantified by image analysis. Cells were untreated or pretreated for three days with BA or RA before being plated onto uncoated (the migration assay) or Matrigel-coated (the invasion assay) polycarbonate membranes. Butyric acid or RA was added to the pretreated cells at the time of plating onto the membranes. After 72 hrs of incubation, cells were fixed, permeablized and stained with Bodipy phallacidin for F-actin, and examined by fluorescent microscopy. The F-actin was divided into two categories, nonfilamentous and filamentous actin. Untreated migratory cells had less filamentous F-actin than BA-pretreated migratory cells (78% decrease, $p<0.05$) and RA-pretreated migratory cells (65% decrease, $p<0.05$). BA-pretreated invasive cells had less nonfilamentous F-actin than RA-pretreated invasive cells (9% decrease, $p<0.05$). Migratory untreated cells had less filamentous actin than invasive untreated cells (26.1% decrease, $p<0.05$). Invasive and migratory cells were capable of bundling actin. Few actin cables were found in noninvasive untreated cells, while actin cables were numerous in nonmigratory untreated cells. In addition, large bundles of actin were predominate in nonmigratory and noninvasive cells, regardless of whether the cells were drug treated or not. The F-actin phenotype is dependent on drug treatment, substrate interactions, migration and invasion.

INTRODUCTION

The cytoskeleton has many different properties essential for normal functioning including maintenance of cell shape, cellular adhesion and cellular migration. The cytoskeleton has been divided into three different classes based on the size and composition of the proteinaceous filaments present in the cytoplasm. They are microfilaments, intermediate filaments and microtubules.

Microfilaments are essential for cell motility and have been shown by fluorescent and transmission electron microscopic (TEM) techniques to be present in the leading edge of motile cells (Lewis et al., 1982; Jockusch et al., 1983). They are composed of F-actin, myosin, filamin, tropomyosin, and alpha-actinin. The organization of F-actin has been inversely correlated with motility (Lewis et al., 1982; Herman et al., 1981). Diffuse actin fluorescence in HeLa cells has been observed in the areas of motile cytoplasm such as leading lamellae and ruffling membrane (Herman et al., 1981). Large bundles of microfilaments, called stress fibers, are lacking in rapidly moving embryonic chick cells, human skin fibroblasts (Lewis et al., 1982), and murine K-1735 melanoma cells (Raz and Geiger, 1982). In addition, motility is inhibited by microfilament disrupting agents such as cytochalasin B (Keller and Zimmermann, 1986).

Microtubules affect cellular migration. Microtubule depolymerizing agents colchicine and vinblastine stimulate random motility (Keller and Zimmermann, 1986) while inhibiting deformability and chemotaxis (Spiro and Mundy, 1980). Immunolabeling has shown that

microtubules stabilize contacts for stress fiber assembly (Rinnerthaler et al., 1988).

Changes in tumor cell morphology, migration rate and invasion rate have been observed in response to BA and RA (McGarvey and Persky, 1989a; McGarvey et al., 1990). An increase or appearance of stress fibers occured in RA-treated F9 teratocarcinoma (Lehtonen et al., 1983), RA-treated rat osteoblastic sarcoma cells (Ng et al., 1985), BA-treated virus transformed (KNRK) fibroblasts (Altenburg et al., 1976), and BA-treated rat hepatoma cells (Borenfruend et al., 1980).

In order for metastasis to occur, tumor cells must invade tissue boundaries and basement membranes at multiple points. Invasion is a three step process that includes: (1) cellular adhesion to a substrate, (2) proteolytic digestion of the substrate, and (3) migration of the cell into the space created by proteolytic digestion (Liotta et al., 1983). Few reports have investigated the role of actin microfilaments in tumor cell migration or invasion. Psuedopodal extension has been observed in migratory and invasive cells (Kramer et al., 1986; McGarvey and Persky, 1989b). Actin filaments of low metastatic human melanoma variants (A375P) become disorganized after invasion through the human amnion basement membrane (Hendrix and Seftor, 1986); however, the cells that had successfully invaded the amnion were enzymatically detached and then plated onto glass slides. Thus, actin analysis was performed on cells that had invaded and attached to a different substrate than originally used.

In our study, the actin cytoskeleton of untreated, BA-treated and RA-treated B16a cells was visualized by fluorescence and quantified by

computer image processing and analysis. Cells on the top and bottom of uncoated and Matrigel-coated polycarbonate (p.c.) membranes were visualized directly by fluorescent microscopy and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Cell Culture: Murine B16a (amelanotic) melanoma cells were obtained from the DCT Tumor Repository (NCI Frederick Cancer Research Facility, Frederick, Maryland). Cells were cultured in Eagle's Minimum Essential Medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and L-glutamine supplemented with sodium pyruvate (110 mg/l) (Sigma, St. Louis, MO), 10% Nu-Serum (Collaborative Research, Bedford, MA), 1% penicillin G-streptomycin sulfate-amphotericin B (Fungizone) (Gibco), MEM non-essential amino acids (10 ml/l) (Gibco), sodium bicarbonate (1.27 g/l) (Sigma), and Hepes (5.96 g/l) (Sigma) (final pH of the medium was 7.3). Cells were cultured in a humidified incubator ($37^{o}C$ in 5% CO_2) and were refed every three days. Cells were removed from flasks with trypsin/EDTA (Sigma) during log phase growth. Cells were injected into 46 day old female C57BL6 mice (Jackson Laboratories, Bar Harbor, ME) either subcutaneously or by lateral tail vein. Subcutaneously-derived B16a cells were used in the migration assay. Lung nodule-derived B16a cells were used in the invasion assay. The derivation of these cells is detailed elsewhere (McGarvey et al., 1990). The cytostatic concentrations of butyric acid (J.T. Baker, Phillipsburg, NJ) and all trans-retinoic acid (Sigma) were chosen from a range of concentrations that were previously shown to be cytostatic (McGarvey and Persky, 1989). The cytostatic concentrations of BA and RA used in all migration and invasion assays were 1.5 mM and 1×10^{-6} M, respectively.

Migration and Invasion Assays: Assays were performed as described

in two previous studies (McGarvey and Persky, 1989; McGarvey et al., 1990). Briefly, B16a cells were untreated or pretreated with BA or RA for three days. In both the migration and invasion experiments, 5 X 10^4 or 1 X 10^3 cells in appropriate media (MEM + NuSerum with or without BA or RA) were plated in each Transwell insert (inner well) (Costar Inc., Cambridge, MA). The matching outer wells contained MEM + NuSerum with or without BA or RA, but without cells. The 1 X 10^3 cell concentration was chosen in order to be able to quantitate the actin of individual nonmigratory and noninvasive cells. Polycarbonate (p.c.) membranes in the migration experiment contained 5 um diameter pores (7.9% of the membrane is represented by pores) and were uncoated. The p.c. membranes in the invasion experiment contained 8 um diameter pores (5.0% of the membrane is represented by pores) and were coated with Matrigel (Collaborative Research, Bedford, MA). The Matrigel was diluted 1:20 in media prior to application to the Transwell inserts in the cold (71 ug protein per 6.5 mm diameter membrane). The duration of the migration and invasion assays was 72 hrs.

Fluorescent Staining: Bodipy phallacidin (Molecular Probes Inc., Eugene, OR) staining of F-actin was performed as described by Barak et al. (1980). In brief, cells on uncoated p.c or Matrigel coated p.c. membranes were rinsed 2x with PBS, fixed with 2% paraforaldehyde in PBS, rinsed with PBS, permeablized with 0.5% Triton X, rinsed in PBS, stained with Bodipy phallacidin, and rinsed again with PBS. Matrigel and cells on top of the p.c. membrane were removed with a cotton swab from some of the p.c. membranes in order to remove background staining (cells on top of the membrane); this removal procedure was performed in

order to permit accurate analysis of the fluorescence of the migratory or invasive cells that were located on the bottom of the p.c. membrane.

Photography and Image Analysis: Cells were photographed with a Leitz Orthoplan microscope utilizing an epifluorescent light source and a number I2 filter block. The fluorescent image was recorded on Kodak Tri-X film at 400 ASA. Exposure time for each negative was determined automatically by the camera system within the films minimum and maximum density. Minimum density is defined as that point where exposure time and light intensity make a first recording on the film. Maximum density is defined as that point where increasing either time or intensity fails to further enhance the density of the image on the film. Increasing exposure time or light intensity past the maximum density point causes the entire negative to turn progressively black (Practical Densitometry, Kodak Publication No. E-59, 1985). Because the intensity of the fluorescence varies with exposure time for the Tri-X film and because film development varies from film strip to film strip, it was necessary to standardize each negative for quantitative image analysis. The morphological depiction (i.e. micrograph) of F-actin was achieved by the optimal exposure for each negative. Thus, because each fluorescent micrograph represents the ideal photograph, it is not possible to compare the actin from one photomicrograph to the next. In short, the fluorescent micrographs are qualitative and nonstandardized (uncorrected) while the image analysis data are quantitative and standardized (corrected).

For the image analysis, each 35 mm negative was subsequently digitized using a Javelin MOS solid state high resolution television

camera interfaced through a Colorado Video Inc. digitizer that was connected to a Princeton Gamma-Tech image analyzer. A total of 90 migratory (30 control, 30 BA-pretreated, and 30 RA-pretreated) and 90 invasive (30 control, 30 BA-pretreated, and 30 RA-pretreated) cells were individually digitized (400 x 512 pixels). A total of 90 nonmigratory (30 control, 30 BA-pretreated, and 30 RA-pretreated) and 90 noninvasive (30 control, 30 BA-pretreated, and 30 RA-pretreated) cells were individually digitized (400 X 512 pixels). For each digitized image, the 256 grey levels were arbitrarily divided into 7 grey levels: 0-87, 88-112, 113-132, 133-157, 158-174, 175-184 and 185-256. Each grey levels was assigned a color. The extracellular background for each negative was standardized by using the intensity control on the Colorado video digitizer until the extracellular background was always within the 175-184 grey level range. Several attempts were usually required to standardize the background. This standardization procedure is an "internal standard protocol" and is a recommended procedure for the PGT image analysis work (personal communication; Princeton Gammma-Tech; Peter Quigley, Product Manager; George Helmstetter, Senior Service Specialist (609-924-7310)). After this standardization procedure for each negative, no data was observed in the 185-256 grey levels. Thus only 6 grey levels remained in the final digitized image. The percent area for each of these 6 grey level was determined for each negative. This initial percent area included both cellular and extracellular data. The grey level group that was extracellular was removed (grey levels 175-184) and new percent areas were calculated based only on cellular grey levels. Five grey level

groups thus represented the cell. Fluorescent intensity was arbitrarily divided into five final grey levels defined as: (1) greatest intensity of stained actin aggregates or actin cables (designated actin 3, grey level 0-87); (2) intermediate intensity of stained actin aggregates or actin cables (designated actin 2, grey level 88-112); (3) least intense stained actin cables, diffuse actin, or actin aggregates (designated actin 1, grey levels 113-132); (4) diffuse actin (grey levels 133-157; no actin cables or aggregates); and cellular background (grey levels 158-174; nonfluorescent nonactin cytosol). The intensely stained actin cables appear to be due to an increase in the number of cables rather than the size of the cables. Figures 1 and 2 illustrate the image analysis protocol.

Measurement of Cell Area: The area of each migratory or invasive cell (180 total) was measured by a Videoplan system (Carl Zeiss Inc.) and analyzed with a measuring histogram software program. The five final grey level groups were converted into area by multiplying the area (um^2) of each cell by the percent area of the actin (um^2/um^2) in each cell.

Statistics: The percent of actin and cell areas of the three grey levels of migratory BA-pretreated cells were individually compared to the percent of actin and areas of untreated or RA-pretreated cells using the either the Welch's tw (t') test unequal variance or the Bechhofer-Dunnett-Krishnaiah-Armitage muliple comparison test (Wilcox, 1987). Both percent of actin and cell area of the three cellular grey levels of migratory RA-pretreated cells were individually compared with the percent actin and areas of untreated cells using the either the

Welch's tw (t') test unequal variance or the Bechhofer-Dunnett--Krishnaiah-Armitage multiple comparison test (Wilcox, 1987). The percent actin and areas of migratory cells were compared separately from invasive cells. Percent actin from similiarly treated migratory and invasive cells were also compared. For example, the nonfilamentous actin was compared between untreated migratory and invasive cells. The percent actin of the three grey levels of nonmigratory untreated, BA-pretreated and RA-pretreated cells were individually compared with the matching percent actin of the three grey levels of similarly treated migratory cells. The percent actin grey levels of invasive and noninvasive cells were similarly evaluated.

A significance level of $p<0.05$ was defined as significant for all experiments.

Transmission Electron Microscopy: Transmission electron microscopy was performed as described in a previous study (McGarvey and Persky, 1989b). Briefly, membranes were fixed for 30 min at 37°C with 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4), rinsed in buffer (3X) for 5 min at 37°C and incubated for 30 min in buffer containing 0.8% $K_3Fe(CN)_6$. Membranes were then incubated for 30 min in buffer containing 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$, rinsed in buffer, rinsed in distilled water (3X), stained en bloc with 2% uranyl acetate (dH_2O) for 60 to 120 min, rinsed in distilled (3X) and dehydrated in ethanol. Membranes were infiltrated into resin (Epon-Araldite), cut out of the Transwell insert, and polymerized. Thin sections were cut, stained in 1% uranyl acetate (in 70% methanol) and lead citrate, and viewed with a Hitachi H-600 microscope at 75 kV accelerating voltage.

RESULTS

The data from the results of the image analysis were simplified as follows. The percent area of background, diffuse actin, and actin 1 were combined into a major group designated nonfilamentous actin. Likewise, the area of background, diffuse actin, and actin 1 were combined.

Quantification of F-actin in Migratory Cells (Table 1): BA-pretreated cells have less nonfilamentous actin than untreated cells (19.2% decrease, $p<0.05$). BA-pretreated cells have less nonfilamentous actin than RA-pretreated cells (13.3% decrease, $p<0.05$). Untreated cells have less actin 2 than RA-pretreated cells (42% decrease, $p<0.05$) and BA-pretreated cells (64% decrease, $p<0.05$). Untreated cells also have less actin 3 than RA-pretreated cells (66% decrease, $p<0.05$) and BA-pretreated cells (83% decrease, $p<0.05$).

Quantification of the Total Area and Area of Actin in Migratory Cells: BA-pretreated cells had more total area than untreated cells (189% increase, $p<0.05$). There was no significant change in area with RA-pretreatment (Fig. 3). BA-pretreated cells had more nonfilamentous actin and filamentous actin (actin 2 and 3) in area than untreated cells (158% increase, $p<0.05$; 601% increase, $p<0.05$; and 701% increase, $p<0.05$, respectively) (Table 2). There was an increase in the area of actin 2 and actin 3 of RA-pretreated cells compared to untreated cells (188% increase, $p<0.05$,; and 272% increase, $p<0.05$, respectively) (Table 2).

Fluorescence, Migration Assay: At a plating concentration of 5 X

10^4 cells, cell processes usually overlapped on top of the uncoated p.c. membrane (Fig. 4A, C, and E). Few F-actin filaments were observed on top of the membrane in untreated or drug-treated cells (Fig. 4A, C, and E). Individual cells were seen on the bottom of the p.c. membrane. F-actin aggregates and stress fibers were evident in untreated migratory cells (Fig. 4B). An organized pattern of F-actin filaments predominates throughout BA-pretreated cells. No F-actin aggregates were seen (Fig. 4D). Parallel F-actin filaments and F-actin aggregates were observed in RA-pretreated cells (Fig. 4F).

Quantification of Actin in Invasive Cells (Table 1): There was no significantly difference in nonfilamentous actin, actin 2 and 3 of untreated cells compared to BA-pretreated cells and RA-pretreated cells. However, there was a significant increase in nonfilamentous actin in RA-pretreated cells compared to BA-pretreated cells (110% increase, $p<0.05$). In addition, there was a significant decrease in actin 2 in RA-pretreated cells compared to BA-pretreated cells (56% decrease, $p<0.05$).

Quantification of the Total Area and Area of Actin in Invasive Cells: BA-pretreated cells had a significantly larger total area than untreated cells (160% increase, $p<0.05$). There was no significant difference in total area between untreated and RA-pretreated cells (Fig. 5). BA-pretreated cells had a larger area of nonfilamentous actin, actin 2 than untreated cells (131% increase, $p<0.05$; and 231% increase, $p<0.05$, respectively) (Table 2). There was a significant decrease in the area of actin 2 and 3 of RA-pretreated cells compared to untreated cells (51% decrease, $p<0.05$; and 34% decrease, $p<0.05$,

respectively). (Table 2).

Fluorescence, Invasion Assay: At a plating concentration of 5 X 10^4 cells, individual cells on top of Matrigel-coated p.c. membranes can not be delineated. Diffuse or filamentous actin observed at different focal points renders high magnification difficult (Fig. 6A, C and E). Untreated cells on top of the membrane have diffuse actin at the cell borders (Fig. 6A). RA-pretreated and BA-pretreated cell processes contain F-actin filaments (Fig. 6C and E). An untreated invasive cell on the bottom of the p.c. membrane has parallel F-actin filaments and F-actin aggregates (Fig. 6B). A dense pattern of F-actin is observed in BA-pretreated invasive cells (Fig. 6D). Stress fibers are observed in RA-pretreated cells (Fig. 6F). Both BA-pretreated and RA-pretreated invasive cells apppear to lack F-actin aggregates, but diffuse staining actin is observed (Fig. 6A and F).

Stress fibers are not apparent in a BA-pretreated invasive cell (Fig. 1). Two pores of the polycarbonate membrane are surrounded with diffuse actin. A ring of organized actin surrounds one of the two pores. F-actin microspikes can be observed within the leading edge of the cell ending at the cell border. A trailing tail can also be seen (Fig. 1).

Comparision of F-Actin between Migratory and Invasive Cells: There was an increase in actin 2 in untreated invasive cells compared to untreated migratory cells (151%, $p<0.05$). There was an increase in actin 2 and 3 in RA-pretreated migratory cells compared to RA-pretreated invasive cells (169%, $p<0.05$; 214% $p<0.05$). There was more actin 3 in BA-pretreated migratory cells than in BA-pretreated

invasive cells (258% increase, $p<0.05$).

Quantification of Actin in Nonmigratory and Noninvasive Cells (Table 3): There was more nonfilamentous actin in RA-pretreated nonmigratory cells than BA-pretreated nonmigratory cells (135% increase, $p<0.05$). There was less actin 2 in nonmigratory RA-pretreated cells than untreated nonmigratory cells (29% decrease, $p<0.05$). Nonmigratory BA-pretreated cells had more actin 3 than untreated or RA-pretreated cells (182% increase, $p<0.05$; 249% increase, $p<0.05$, respectively). There was more actin 3 in BA-pretreated noninvasive cells than untreated noninvasive cells (167% increase, $p<0.05$).

Comparison of F-Actin between Nonmigratory and Migratory Cells (Tables 1 and 3): Migratory untreated cells had more nonfilamentous actin than untreated nonmigratory cells (147% increase, $p<0.05$). There was more actin 2 and 3 in untreated nonmigratory cells compared to untreated migratory cells (425% increase, $p<0.05$; 850% increase, $p<0.05$, respectively). There was more nonfilamentous actin in migratory BA-pretreated cells than nonmigratory BA-pretreated cells (137% increase, $p<0.05$). Nonmigratory BA-pretreated cells contained more actin 3 than migratory BA-pretreated cells (266% increase, $p<0.05$). There was more actin 2 and 3 in RA-pretreated nonmigratory cells compared to RA-pretreated cells (174% increase, $p<0.05$; 211% increase, $p<0.05$, respectively).

Comparison of F-Actin between Noninvasive and Invasive Cells (Tables 1 and 3): Untreated invasive cells had more nonfilamentous actin than untreated noninvasive cells (125% increase, $p<0.05$). There

was more actin 2 and 3 in untreated noninvasive cells compared to untreated invasive cells (237% increase, $p<0.05$; 342% increase, $p<0.05$, respectively). BA-pretreated invasive cells had more nonfilamentous actin than BA-pretreated noninvasive cells (127% increase, $p<0.05$). Noninvasive BA-pretreated cells contained more actin 2 and 3 than invasive BA-pretreated cells (153% increase, $p<0.05$; 381% increase, $p<0.05$). RA-pretreated invasive cells had more nonfilamentous actin than RA-pretreated noninvasive cells (137% increase, $p<0.05$). There was more actin 2 and 3 in RA-pretreated noninvasive cells than RA-pretreated invasive cells (314% increase, $p<0.05$; 664% increase, $p<0.05$, respectively).

Fluorescence, Nonmigratory and NonInvasive Cells: Individual cells were observed on top of uncoated (Fig. 7A, C, and E) and Matrigel-coated (Fig. 7B, D, and F) p.c. membranes at a plating concentration of 1×10^3 cells. Prominent stress fibers are seen in untreated nonmigratory cells (Fig. 7A). In contrast, few stress fibers were visualize in untreated noninvasive cells (Fig. 7B). Organized stress fibers can be observed in the drug-treated cells on top of either substrate (Fig. 7C, D, E, and F) However, RA pretreated nonmigratory cells contain paranuclear actin staining not observed in RA-pretreated noninvasive cells (Fig. 7E and F)

Transmission Electron Microscopy of Migratory and Invasive Cells: This investigation observed the cytoskeleton of migratory and invasive cells above, below and within the pores of the polycarbonate membrane (Figs. 8-10). Higher magnification of the cytoplasm within the pore of migratory or invasive cells reveals microtubules and cortical actin.

The microtubules are parallel to the apparent movement of the cell (Figs. 8-9). Cortical actin is most often observed adjacent to the Matrigel, the p.c. membrane, and at cell margins in noninvasive and invasive BA-pretreated cells (Fig. 10).

DISCUSSION

This report quantitatively compared the actin cytoskeleton of untreated and drug-treated cells that had or had not migrated or invaded in a three dimensional assay. There were two major goals of this study. The first purpose was to quantitate nonfilamentous and filamentous actin between noninvasive and invasive cells as well as between nonmigratory and migratory cells. The second purpose was to describe morphologically F-actin when modulated by drug treatment, substrate, and the processes of migration and invasion.

Past reports have compared cells on two dimensional substrates such as glass or plastic. Semiqualitative analysis of immunolabelled or phalloidin labelled cytoskeletons has been limited to studies of motility or chemotaxis (Volk et al., 1984; Anderson et al., 1982). Flow cytofluorimetry has been used to compare total F-actin fluorescence between chemotactically active and nonactive polymorphonuclear neutrophils (PMNs) (Howard and Meyer, 1984) and has shown an increase in total actin in BA-treated hepatoma cells (Ryan et al., 1987). The ability to quantitate F-actin by fluorescence is based on the unique stoichiometric binding of F-actin to phallacidin (1:2) (Miyamoto et al., 1986).

Both area of actin and percent of actin within cells were computed in this study. The area of actin data showed that BA-pretreated migratory and invasive cells were larger in size and had more nonfilamentous actin and actin 2 than untreated migratory and invasive cells. In contrast, the percent of actin demonstrated differences in

actin groups that were not based on cell size.

Cell shape, degree of confluency, and cell spreading affect cytoskeleton visualization (DeMey et al., 1978; Wolin and Kucherlapati, 1979). In my study, individual cells on top of uncoated or Matrigel-coated p.c. membranes could not be delineated when the cell plating density was 5 X 10^4, and thus actin fluorescence was quantified at the lower density (1 X 10^3).

Substrate interactions are important in the morphology of actin filaments. The actin cytoskeleton appears nondistinct in untreated or treated cells plated at 5 X 10^4 cells on top of uncoated p.c. membranes. This was in contrast to the morphology of the F-actin cytoskeleton of cells plated at 5 X 10^4 cells on top of Matrigel-coated p.c. membranes. The actin fluorescence of untreated cells on top of Matrigel appeared similiar to the actin morphology of epithelial cells in situ where F-actin fluorecence is largely confined to cell periphery (Friedman et al., 1984) (Fig. 6A). In addition, the actin cytoskeleton was still diffuse in untreated cells plated at a concentration of 1 X 10^3 cells (Fig. 7B). Extensive actin filaments, on the other hand, were observed in BA or RA-treated cells on top of the Matrigel at either plating concentration (Fig. 6C and E, Fig. 7D and F). Restoration of stress fibers and adhesion plaques has been reported in some transformed cells when grown on fibronectin (Ali et al., 1977; Willingham et al., 1977). Glass or plastic substrates allow for the formation of stress fibers which are generally absent from most cells in situ (Burridge et al., 1987). Nuclear F-actin aggregates or peripheral stress fibers observed in glass grown untreated B16a cells

(Persky and McGarvey, 1990) were not observed in migratory or invasive untreated B16a cells. Therefore, F-actin expression appeared to be dependent on both the cell substrate and cell activity. Thus, the value of comparing noninvasive and invasive cells can be questioned, since noninvasive cells were on a Matrigel substrate and the invasive cells were attached to an uncoated p.c. membrane.

The actin morphology of the BA-treated cell in Figure 2A appears to typical of migratory cells (Lewis et al., 1982; Rinnerthaler et al., 1988). Prominent stress fibers were not present. Instead, diffuse actin was prominent with microspikes bundles at the leading edge and a retracting tail. This morphology was similiar to that observed for migratory fibroblasts (Lewis et al., 1982; Rinnerthaler et al., 1988). On the other hand, nonmigratory fibroblasts have prominent stress fibers (Lewis et al., 1982). Large stress fibers seem to have a structural role rather than a migratory role. I observed an increase actin 3 in all nonmigratory and noninvasive cells compared to migratory and invasive cells. The most striking increase in stress fibers was seen in untreated nonmigratory cells compared to migratory untreated cells (Fig. 7A).

Transmission electron microscopy of untreated and drug treated cells demonstrated parallel bundles of microtubules and intermediate filaments were observed by TEM in the pseudopods that were within the pores of the p.c. membrane. Microtubules, intermediate filaments, and cortical actin bundles are known to be involved in migration and invasion. Anti-microtubule agents such as colchicine inhibit deformability (Daughaday et al., 1981). Both deformability and

motility are necessary for migration through a porous membrane. Chemotactic activation of PMNs has been shown to increase the average length of microtubules parallel to the direction of cell migration without affecting the number of microtubules per cell (Anderson et al., 1982).

Changes in the cytoskeleton may affect the organization, distribution, and amount of actin or tubulin protein. Ryan and Higgins (1988) found by biochemical analysis that total actin (globular and filamentous actin) represented only 5.45% of total cellular protein in transformed fibroblasts and 18.05% of total cellular protein in BA-treated transformed fibroblasts. I compartmentalized the F-actin fluorescence according to fluorescent intensity and showed that cortical actin is prominent in BA-pretreated noninvasive and invasive cells (Fig. 10). Obviously, the percents of F-actin presented here represented the compression of a three dimensional structure into two dimensions. This compression is seen in Fig. 6D by the overlapping of actin structures. Confocal microscopy would allow for optical sectioning through the cell and can accurately quantify actin at varying cell heights. An increase in actin close to the substrate, as viewed with confocal microscopy, has been reported to be indicative of highly migratory cells (Hay, 1985).

Fluorescent microscopy (Figs. 4 and 6) and image analysis data show that migratory and invasive B16a cells organize actin into cables. The organization of actin is most prominent in invasive untreated, migratory RA pretreated and migratory/invasive BA-pretreated cells. The ability of a cell to organize actin into a structural component of

the cytoskeleton or into a intergral part of the migratory process may predict the migratory/invasive nature of the cell. My invasion data are in contrast to the findings of Hendrix and Seftor (1986), although the differences may be attributed to the protocols used. Hendrix and Seftor studied actin filaments after the invasive cells were detached from the amnion and then allowed to attachment to a glass substrate. They used a different cell line and they utilized the human amnion rather than Matrigel as the invasive barrier. Their study was significant in that the morphology of actin in low metastatic cells that were invasive for the amnion appeared similar to the morphology of high metastatic cells that were noninvasive for the amnion.

Motile fibroblasts possess diffuse actin (Lewis et al., 1982). Interferon treatment of transformed cells restores or increases the number of the stress fibers as well as inhibits tumor cell motility on glass (Pfeffer et al., 1980; Bourgeade et al., 1981). Thus, migratory and invasive cells would be expected to contain only diffuse F-actin. However, migratory and invasive cells contained varying amounts of filamentous actin. Migratory and invasive BA-pretreated cells contain a complex organization of actin filaments compared to migratory and invasive untreated cells. There was a significant increase in actin organization of migratory RA-pretreated Bl6a cells as compared to migratory untreated cells. The increase in F-actin organization would be indicative of a nonmigratory phenotype. In contrast to the migration data, I did not observe an increase in F-actin organization in RA-pretreated invasive cells as compared to untreated invasive cells. In addition, it would be expected that BA-pretreated cells

would be less invasive and migratory than untreated cells, but the converse has been shown (McGarvey and Persky, 1989; McGarvey et al., 1990). Therefore, the function of filamentous actin with respect to motility, migration, and invasion still has to be elucidated.

A shift in the pool of actin from G-actin (globular actin) to F-actin (filamentous actin) has been reported in chemotactically activitated neutrophils (Howard and Meyer, 1984). My study was limited in that I could not determine if there was a shift in the pool of G to F-actin, or measure total actin. It has been noted that BA-treatment of transformed KNRK cells increases the amount and organization of F-actin. However, BA-treatment of tranformed KNRK cells does not completely restore actin to the pattern observed in untransformed KNK cells (Ryan and Higgins, 1988). My study only quantitated the organization of F-actin. Both total actin and F-actin organization are probably important for motility.

An important question in my study is whether F-actin subgroups are selected by migratory and invasive cells. The increase in certain F-actin subgroups in invasive untreated cells compared to untreated migratory cells could indicate that a certain F-actin phenotype is necessary for invasion. In addition to the selective pressure of invasion on F-actin subgroups, F-actin subgroups are modified by drug treatment. In addition, drug treatment may affect a selected cytoskeletal phenotype before or after the migratory and invasive process.

The image analysis system is based on fluroescent intesity of the F-actin, not on the organization of the actin. Thus, image analysis

does not distinguish between intensely stained F-actin aggregates and cables that were observed in migratory and invasive untreated cells. Only F-actin cables were observed in BA-pretreated cells. Therefore, there were more changes that took place with drug treatment than could be quantitated by image analysis. These differences may be important to the enhanced migratory and invasive rates induced by BA.

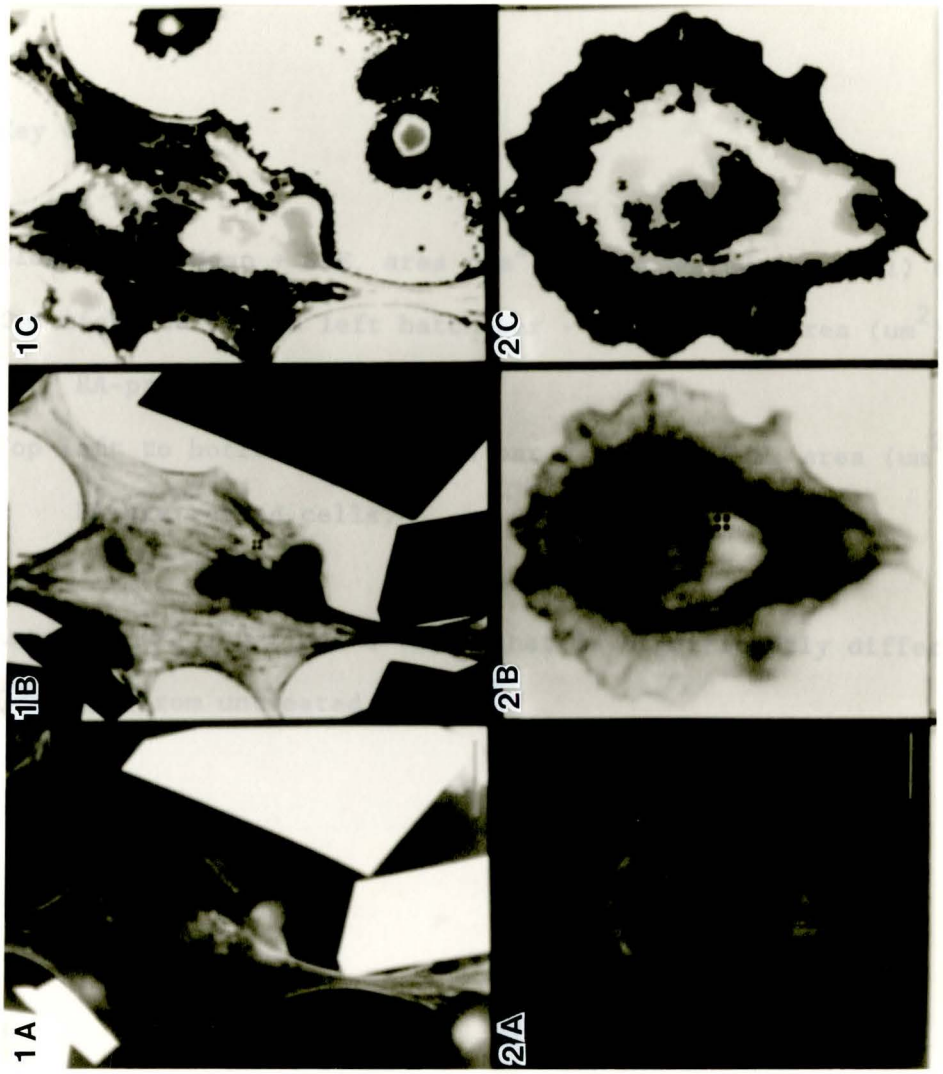
Other cytoskeletal components may function in conjunction with F-actin to regulate migration and invasion. Butyric acid or RA may up or down regulate the production of other cytoskeletal proteins or effect their interaction with F-actin. Regulation of actin polymerization, including gelation, bundling, capping, severing and stabilizing of actin filaments, is a known function of numerous actin-binding proteins (Weeds, 1982). It is possible that each actin-binding protein is necessary in cell motility, but little is known about most of these proteins. One gel-bundling protein, alpha-actinin, has been localized to both stress fibers and to the leading lamellae of motile V2 carcinoma cells (Jockusch et al., 1983). Other actin-binding proteins, such as tropomyosin and myosin that have a role in muscle tissue contractility, are not found in the motile leading edge (Jockusch et al., 1983). Ryan and Higgins (1987) found that BA-treated transformed fibroblasts (KNRK) had a three fold increase in total cellular actin, detergent resistant actin, and alpha-actinin. Linder et al. (1981) observed an increase in synthesis of tropomyosin in RA-treated F9 teratocarcinoma cells. An increase in alpha-actinin or tropomyosin may play a role in migration of B16a cells. Further study must be done on the role of actin binding

proteins, and the modulation of migration by BA or RA treatment.

In conclusion, this study reports on F-actin in nonmigratory, migratory, noninvasive, and invasive cells. The isolation of migratory and invasive cells in the Transwell chamber may allow for future quantitation of changes in the cytoskeleton by biochemical analysis or sensitive radioimmunoassays (Bernal et al., 1983; Ball et al., 1986). Further work utilizing confocal microscopy, monoclonal antibodies and biochemical techniques may help evaluate the mechanisms involved in cell migration and invasion.

Figure 1 - 2.

Fluorescent micrographs (1A and 2A), the digitized (1B and 2B) and the color enhanced images (1C and 2C) of two invasive cells. Figures 1C and 2C illustrate the extracellular background (deep grey), nonfilamentous background (black), least intense filamentous actin (grey), and most intense filamentous actin (white). See the Material and Methods for further detail.


1A
1B
1C
2A
2B
2C

Key to Fig. 3

Clear bar - Mean $\pm$ S.E. area (um^2) of untreated (control) cells.

Top right to bottom left hatch bar - Mean $\pm$ S.E. area (um^2) of RA-pretreated cells.

Top left to bottom right hatch bar - Mean $\pm$ S.E. area (um^2) of BA-pretreated cells.

Asterisk (*) - Defines a value that is significantly different ($p<0.05$) from untreated cells.

Figure 3.

Total area of untreated, RA-pretreated and BA-pretreated migratory cells.

AREA OF MIGRATORY CELLS

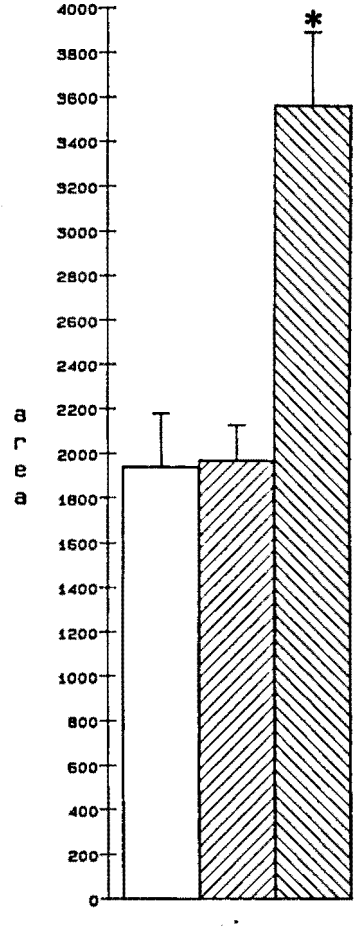
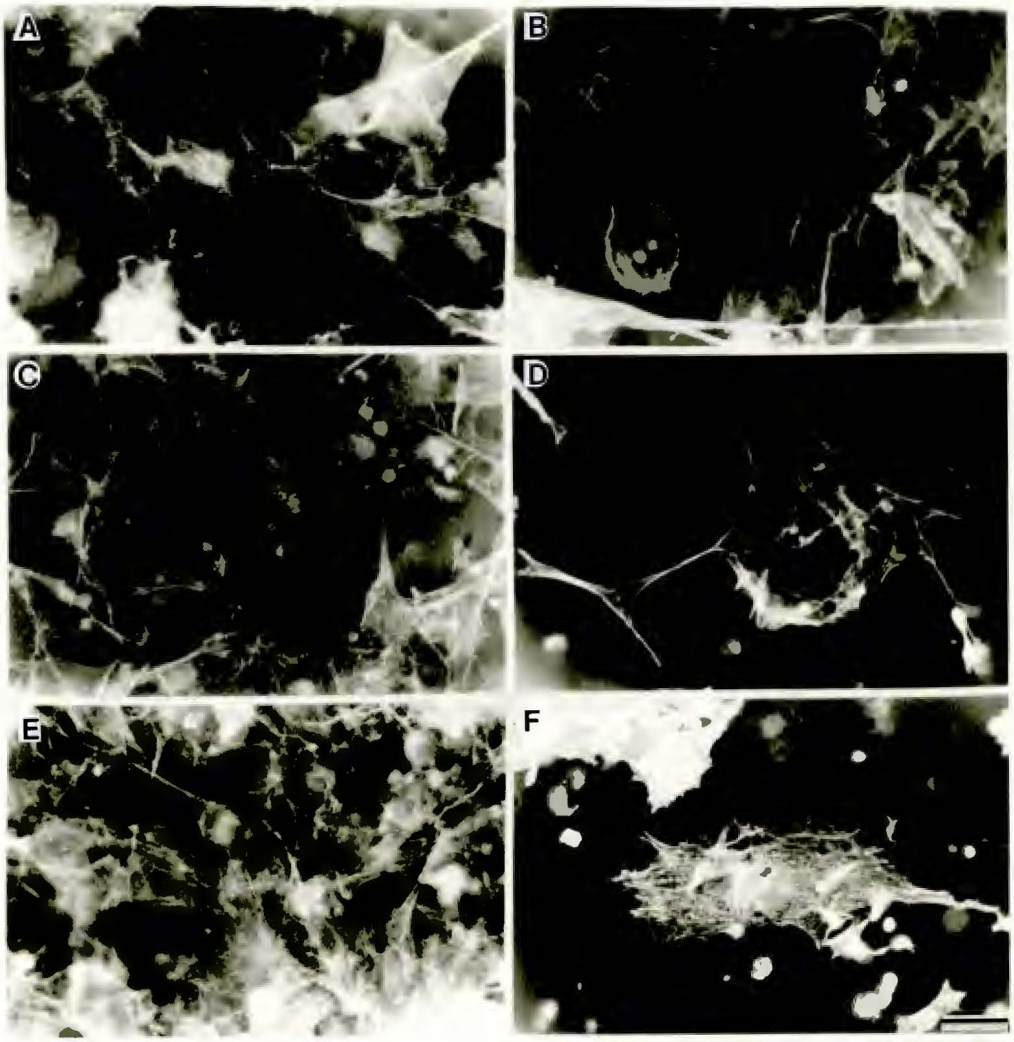


Figure 4.

Flourescent micrographs of cells on the top (A,C,E) and bottom (B,D,F) of uncoated p.c. membrane. Labels A and B are for untreated cells, C and D are for BA-pretreated cells, and E and F are for RA-pretreated cells. Bar = 20 um.


A
B
C
D
E
F

Key to Fig. 5

Clear bar - Mean $\pm$ S.E. area (um^2) of untreated (control) cells.

Top right to bottom left hatch bar - Mean $\pm$ S.E. area (um^2) of RA-pretreated cells.

Top left to bottom right hatch bar - Mean $\pm$ S.E. area (um^2) of BA-pretreated cells.

Asterisk (*) - Defines a value that is significantly different ($p<0.05$) from untreated cells.

Figure 5.

Total area of untreated, RA-pretreated and BA-pretreated invasion cells.

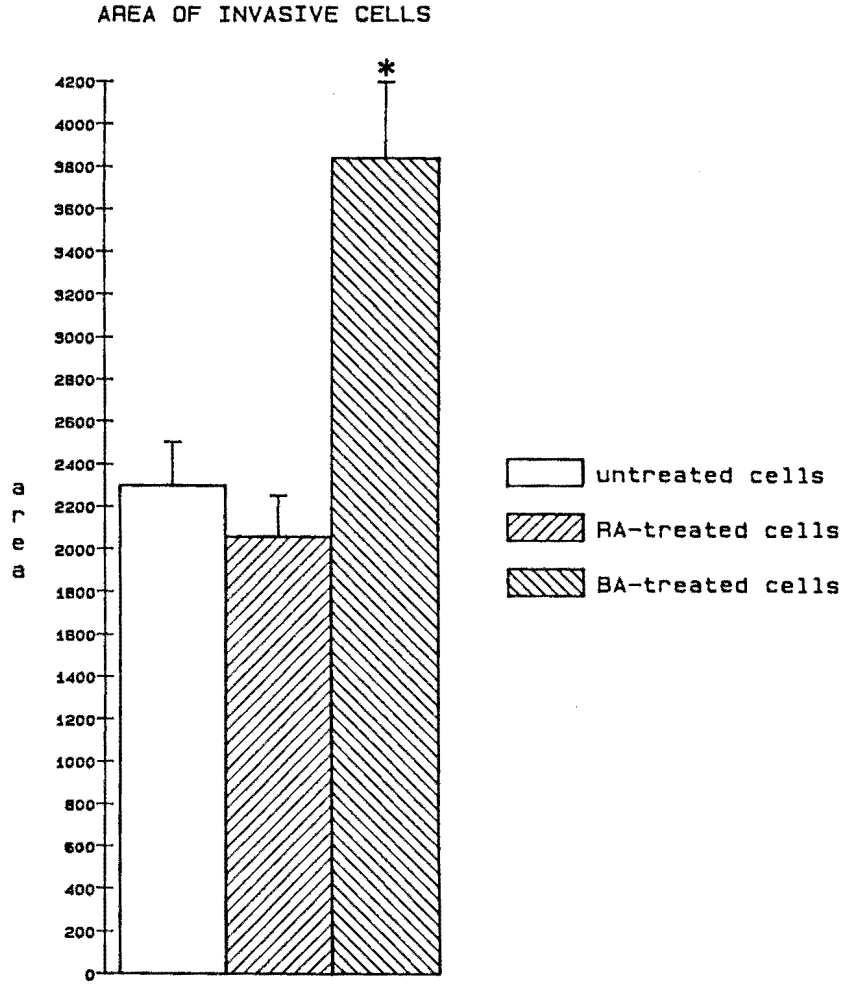

AREA OF INVASIVE CELLS
4200
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
untreated cells
RA-treated cells
BA-treated cells

Figure 6.

Flourescent micrographs of cells on the top (A,C,E) and bottom (B,D,F) of Matrigel-coated p.c. membranes. Labels A and B are for untreated cells, C and D are for BA-pretreated cells, and E and F are for RA-pretreated cells. Bar = 20 um.

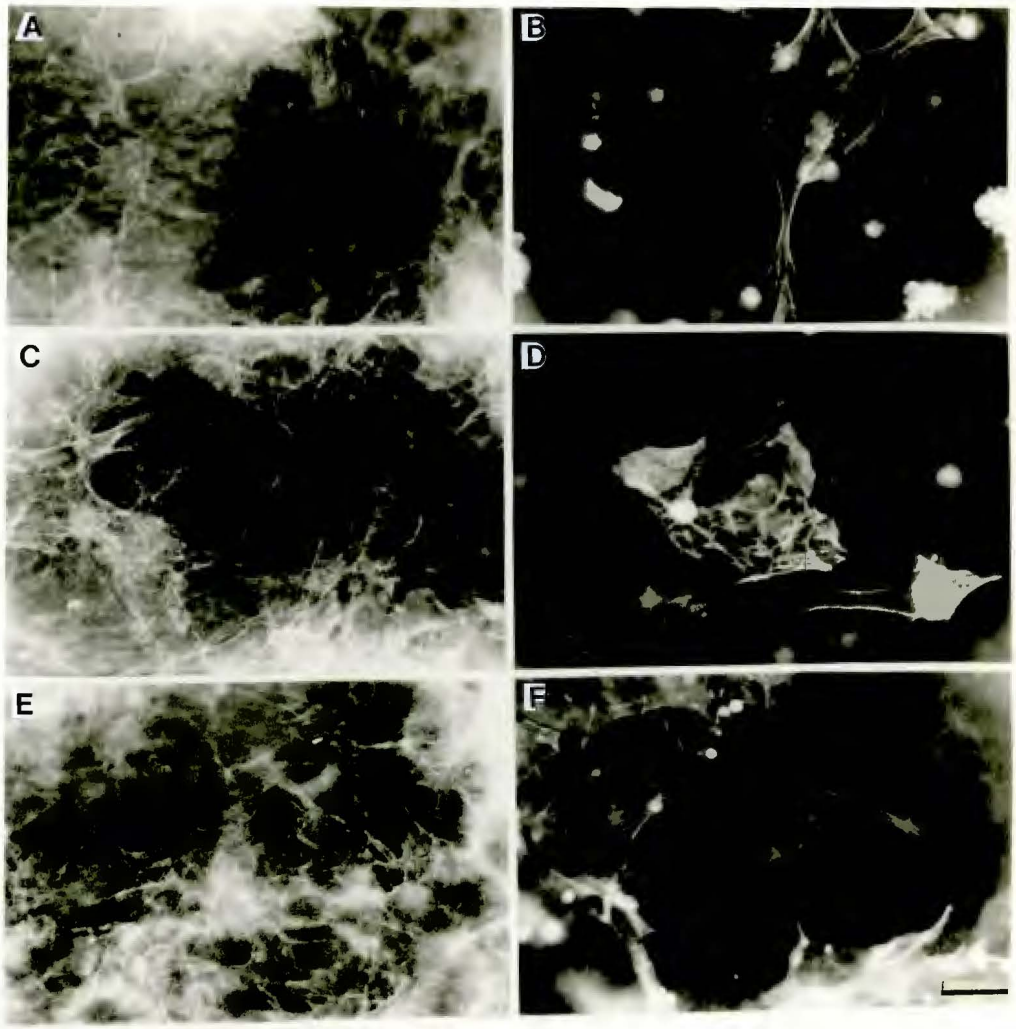
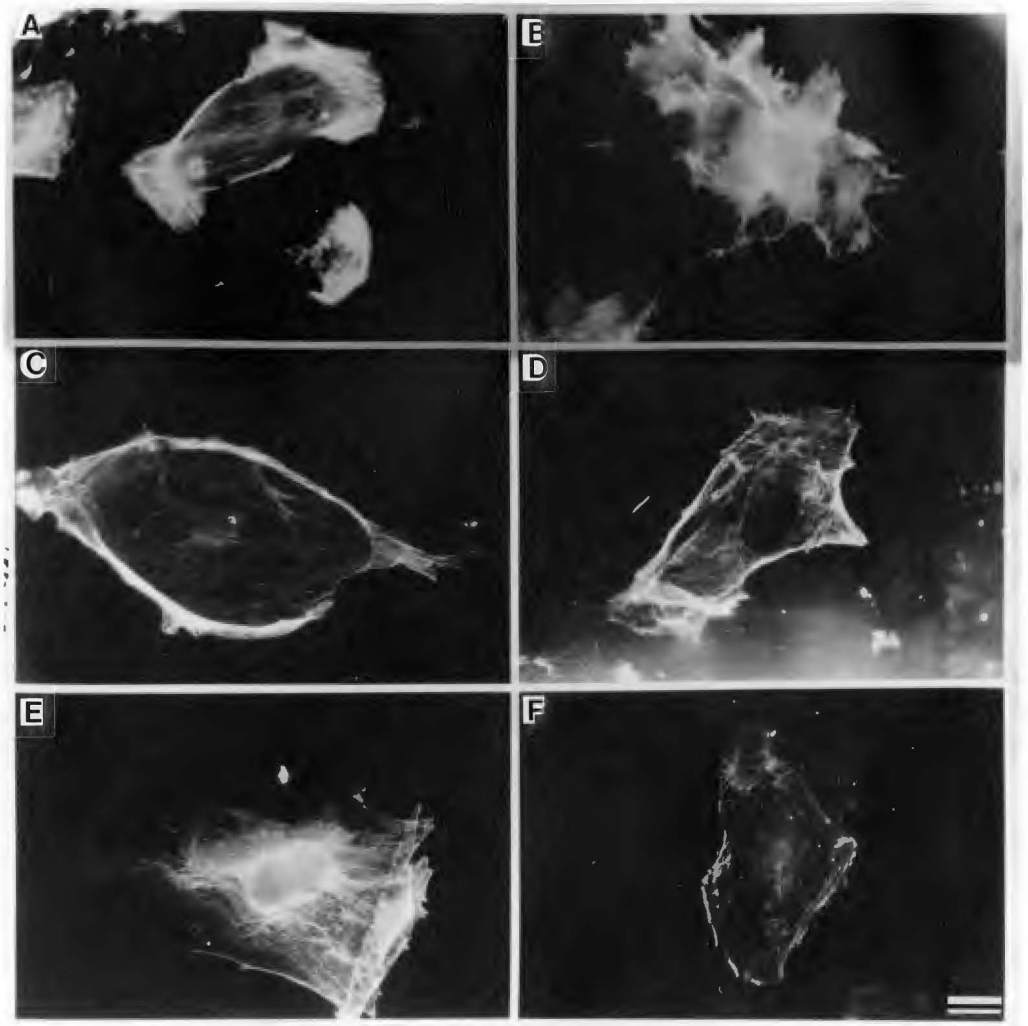

A
B
C
D
E
F

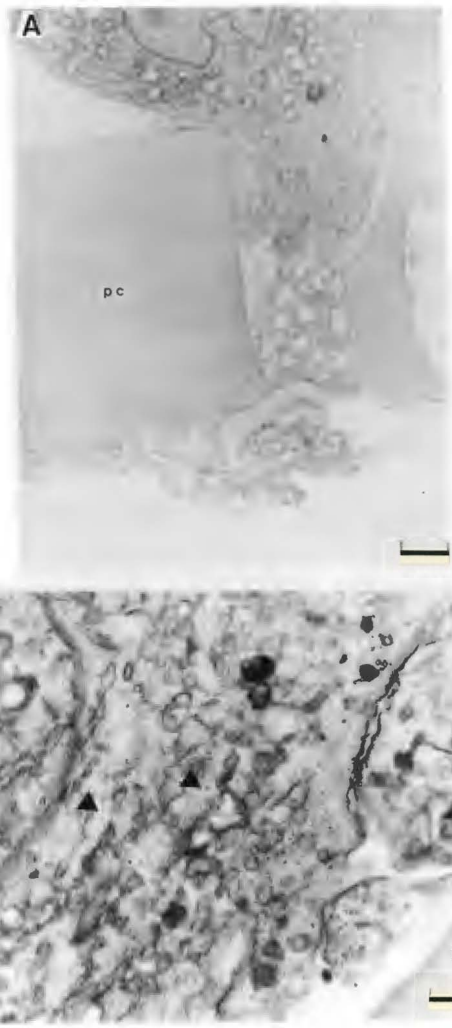
Figure 7.

Fluorescent micrographs of cells on top of uncoated (A,C,E) and Matrigel-coated (B,D,F) p.c. membranes. Labels A, B are for untreated cells, labels C, D are for BA-pretreated cells, and labels E and F are for RA-pretreated cells. Bar = 20 um.


A
B
C
D
E
F

Figures 8A and B.

Transmission electron micrographs of a migratory untreated cell within a pore of the p.c. membrane (p.c.). Cortical actin (a) and microtubules (arrow) are found in the cytoplasm within a pore. Fig. 37A, Bar = 2 um. Fig. 38B, Bar = 1 um.


A
pc
B
pc

Figures 9A and B.

Transmission electron micrographs of untreated cells on the top, within and on the bottom of a Matrigel (m) coated p.c. membrane (p.c). Cortical actin (a) and microtubules (arrows) are found in the cytoplasm within the pore. Fig. 9A and B, Bar = 1 um.

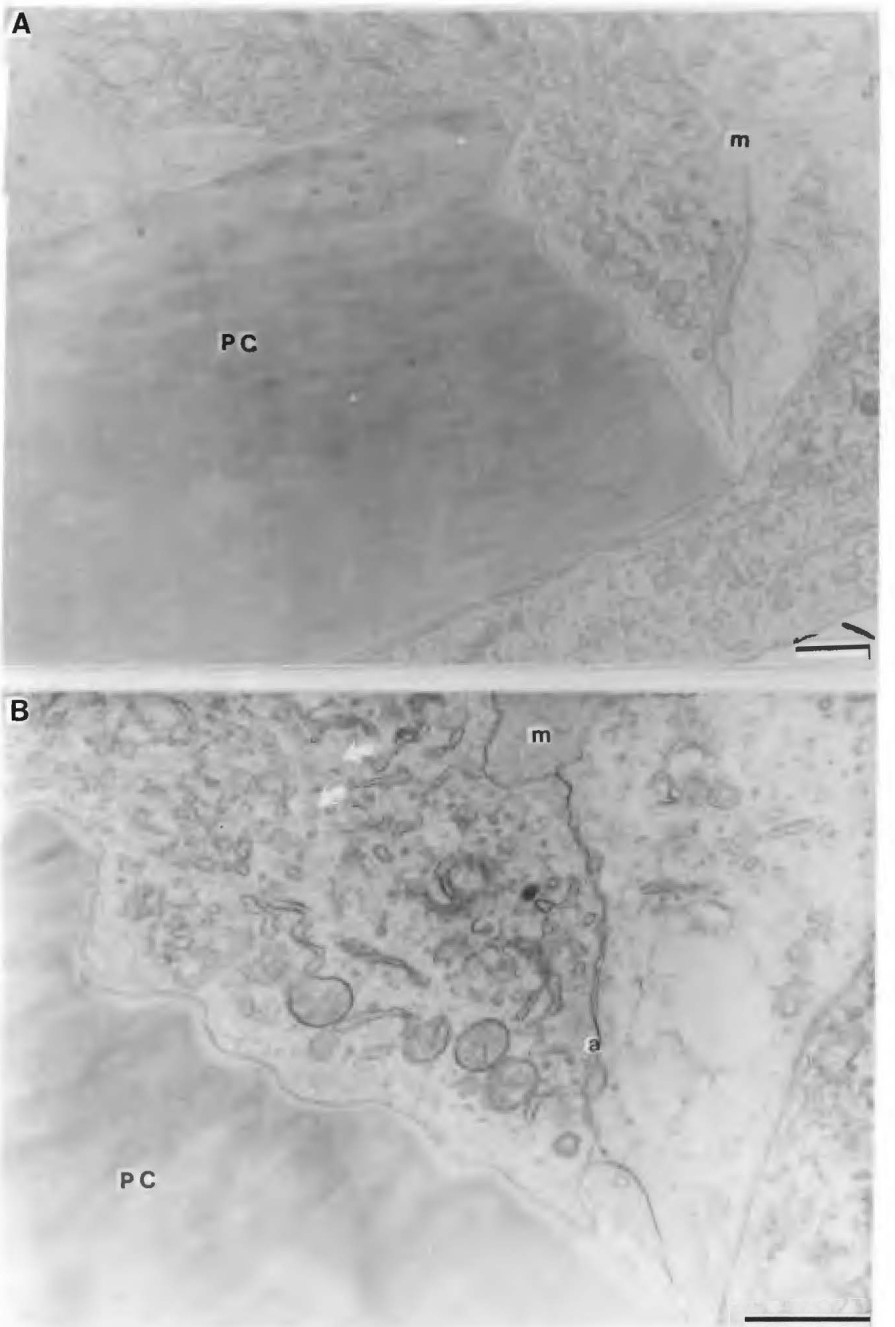

A
m
PC
B
m
a
PC

Figure 10.

Transmission electron micrograph of BA-pretreated cells on top of Matrigel (m) and on the bottom of a p.c. membrane (p.c.). Cortical actin (arrows) is found in both non-invasive and invasive cells. Bar = 2 um.

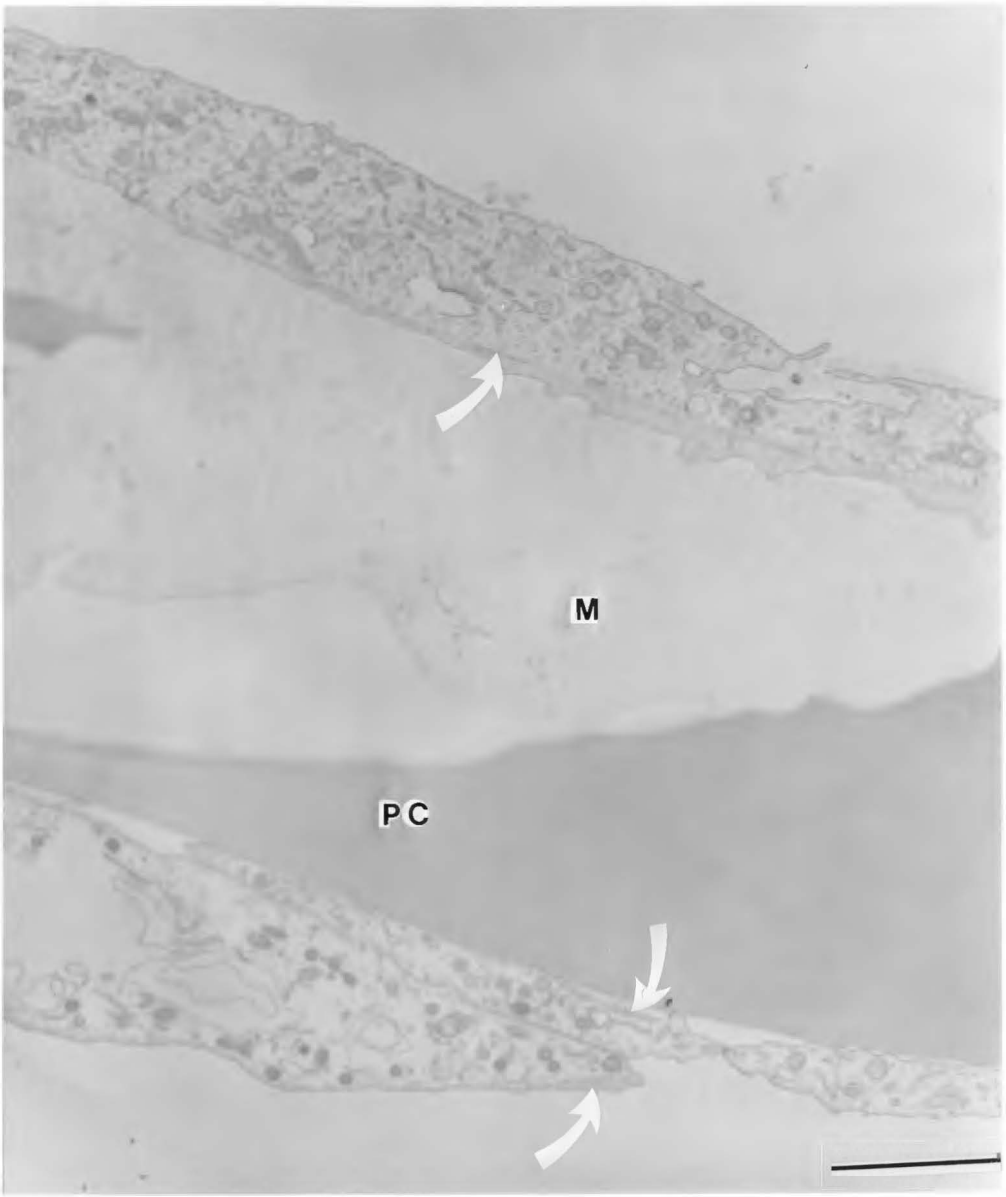

M
PC

TABLE 1.

The percent of the form of F-actin in untreated, BA-pretreated, and RA-pretreated migratory and invasive cells. For details see the Results.

TREATMENT/CONDITION	FORM OF ACTIN (%) N.F. X ± S.E.	L.I.F. X ± S.E.	M.I.F. X ± S.E.
UNTREATED/MIGRATORY	92.6 ± 1.0	5.7 ± 0.9	1.6 ± 0.3
BA-TREATED/MIGRATORY	74.8 ± 2.8*	16.0 ± 1.9*	9.3 ± 1.3*
RA-TREATED/MIGRATORY	86.3 ± 1.8**	9.8 ± 1.1*	4.7 ± 0.9*
UNTREATED/INVASIVE	88. 6 ± 1.8	8.6 ± 1.5	2.4 ± 0.6
BA-TREATED/INVASIVE	83.1 ± 1.6	13.2 ± 1.4	3.6 ± 0.7
RA-TREATED/INVASIVE	91.7 ± 1.3**	5.8 ± 0.9**	2.2 ± 0.4

N.F. - Nonfilamentous actin
L.I.F. - Least intense filamentous actin (Actin 2)
M.I.F. - Most intense filamentous actin (Actin 3)

* Significant $p < 0.05$ level, untreated vs. drug-treated cells.
** Significant $p < 0.05$ level, BA-treated vs. RA-treated cells.

TABLE 2.

The area (um^2) of the form of F-actin in untreated, BA-pretreated, and RA-pretreated migratory and invasive cells. For details see the Results.

AREA OF F-ACTIN

TREATMENT/CONDITION	N.F. X ± S.E.	L.I.F. X ± S.E.	M.I.F. X ± S.E.
UNTREATED/MIGRATORY	2013.0 ± 179.5	110.3 ± 19.3	35.4 ± 9.9
BA-TREATED/MIGRATORY	3172.0 ± 299.8*	662.4 ± 109.3*	248.1 ± 41.0*
RA-TREATED/MIGRATORY	1900.0 ± 182.6**	207.3 ± 28.9*	96.6 ± 22.8*
UNTREATED/INVASIVE	1790.0 ± 214.8	218.3 ± 42.70	49.5 ± 12.4
BA-TREATED/INVASIVE	2650.0 ± 273.7*	505.3 ± 72.2*	142.5 ± 34.0
RA-TREATED/INVASIVE	1673.0 ± 121.9**	107.4 ± 16.7*	32.6 ± 5.9*

N.F. - Nonfilamentous actin
L.I.F. - Least intense filamentous actin (Actin 2)
M.I.F. - Most intense filamentous actin (Actin 3)

* Significant $p < 0.05$ level, untreated vs. drug-treated cells.
** Significant $p < 0.05$ level, BA-treated vs. RA-treated cells.

TABLE 3.

The percent of the form of F-actin in untreated, BA-pretreated, and RA-pretreated nonmigratory and noninvasive cells. For details see the Results.

TREATMENT/CONDITION	FORM OF ACTIN (%) N.F. X ± S.E.	L.I.F. X ± S.E.	M.I.F. X ± S.E.
UNTREATED/NONMIGRATORY	62.8 ± 3.4	24.2 ± 2.3	13.6 ± 1.8
BA-TREATED/NONMIGRATORY	54.5 ± 3.2	20.7 ± 1.4	24.7 ± 2.6*
RA-TREATED/NONMIGRATORY	73.5 ± 2.5**	17.1 ± 1.5*	9.9 ± 1.4**
UNTREATED/NONINVASIVE	70.9 ± 2.4	20.4 ± 2.2	8.2 ± 1.0
BA-TREATED/NONINVASIVE	65.3 ± 2.8	20.2 ± 1.9	13.7 ± 1.4*
RA-TREATED/NONINVASIVE	67.0 ± 2.7	18.2 ± 1.1	14.6 ± 2.3

N.F. - Nonfilamentous actin
L.I.F. - Least intense filamentous actin (Actin 2)
M.I.F. - Most intense filamentous actin (Actin 3)

* Significant $p < 0.05$ level, untreated vs. drug-treated cells.
** Significant $p < 0.05$ level, BA-treated vs. RA-treated cells.

CHAPTER VII

GENERAL DISCUSSION

I have studied the affect of two antitumor agents, butyric acid (BA) and all trans-retinoic acid (RA), on the proliferation, adhesion, migration, invasion, cytoskeleton, and experimental metastasis of murine B16a melanoma cells. Clinical application of BA and RA is increasing despite limited knowledge on how these agents perturb cellular migration and invasion. I have discussed within in chapters IV, V and VI some of the mechanisms behind the the affect of BA and RA on tumor cells. The mechanism of action of BA and RA at the molecular level needs further study.

Phenotypic Drift and Metastasis

Phenotypic diversity and tumor cell progression are important parts of the metastatic process. Tumor cell progression is defined as the evolution of tumor cells through a combination of genetic instability and host selection. Progression produces tumor cells with enhanced autonomy, survival and growth characteristics. The ultimate success of chemotherapy depends on the ability to eliminate rare, highly metastatic cell subpopulations. Elimination of the majority of tumor cells by cytotoxic drugs can create a new group of subclones that are drug resistant and phenotypically unstable. Long term cultures with BA-treated cells have not shown typical signs of drug resistance such as failure to inhibit proliferation. In fact, these cultures have shown further phenotypic changes including enhanced cell spreading. A substantial increase in the invasion or migration rates was noted if the inhibition of proliferation induced by BA was factored into these rates. In addition, I have shown in the migration and invasion assays, have shown that increasing the time of BA incubation further enhances

migration and invasion. This was not the case in the invasion assay with RA-treated and RA-pretreated cells. The limited effect of RA on the invasion of B16a cells may be due to the low invasive capability of B16a cells, since RA-pretreatment of K-1735 cells (a highly invasive cell line) reduced invasion. Radical changes in morphology, eg. cell size were not observed with RA-treatment. The amount of actin in nonmigratory and invasive RA-pretreated cells was significantly less than nonmigratory and invasive untreated cells, respectively. How this information may be related to the decrease in migration and invasion by RA-pretreated cells is open to speculation. Two factors seem to operate to control movement in cells. These are the F-actin to G-actin ratio (an increase in F-actin is indictative of an increase in migration) and the organization of F-actin (higher forms of organization are indicative of a decrease in migration). Both factors seem to operate with RA-pretreated cells. Nonmigratory RA pretreated cells have more total F-actin and increased organization of F-actin than migratory cells. The questions that remain are whether there is a decrease in total actin (globular and filamentous) or if there are changes in actin binding proteins that regulate actin polymerization.

The genetic instability of metastatic tumor cells has been reported in vitro and in vivo. I have observed that the migration rate of untreated B16a cells decreased with increasing passage of cells in culture (McGarvey and Persky, 1989b). I have also observed that no extrapulmonary metastasis occurred with tail vein injection of a mixture of lung nodule-derived and subcutaneously-derived B16a cells (McGarvey et al., 1990). In contrast, Honn et al. (1981) have reported

liver and spleen metastases from tail vein injection of B16a cells. There may a link between decreased migration in the Transwell system and a decrease in extrapulmonary metastasis. Decreased migration of subcutaneously-derived cells compared to early passage cells may be linked to decreased deformability of the cells through the lung capillaries. Subtle changes in genome probably account for these changes.

Phenotypic drift of the B16 melanoma cell line has been observed (Poste et al., 1981; Stackpole, 1983). There was interlesional heterogeneity in the early stages of growth in experimental metastatic assays followed by intralesional heterogeneity (Poste et al., 1981). Before 25 days, cells are homogeneous within lesions. Progressive growth of the nodules gave rise to clones with differing metastatic capability (Poste et al., 1981). Stackpole (1983) has reported that the B16 melanoma cell line was not stable in culture or in serial subcutaneous passage (over 30 monthly generations). During this time, metastatic activity of the original tumor, which was intially slight, was lost by generation 10 and was restored by generation 18. Four different phenotypes were observed. Some lines produced both spontaneous and experimental metastases, some lines produced spontaneous metastases only, some lines produced experimental colonies only, while other lines produced neither spontaneous nor experimental metastases. Phenotypic drift is not exclusive to the B16 cell line (for review see Welch and Tomasovic, 1985). Other tumor cell lines reported to drift with respect to metastatic rate are the murine K1735 melanoma (Talmadge et al., 1984) and clones of the 13762NF mamamary

adenocarcinoma (Welch et al., 1984). Schirrmacher (1980) has reported that the ESb lymphoma cell line has extremely high rates of drift to nonmetastatic behavior. Therefore, care must be taken in evaluating both in vitro and in vivo using metastatic tumor cells. Because of phenotypic drift, I performed tail vein and subcutaneous injections before and after all in vitro studies in order to verify that the cells were tumorigenic and metastatic.

Cellular Migration

The fundamental question in cellular migration is "what is the driving force behind cellular motility?" Clearly actin has a major role, while myosin probably does not. Certain forms of actin especially actin 3, are indicative of nonmigratory untreated or drug treated B16a cells. The actin morphology of BA-pretreated cells appear to be an example of a nonmigratory cell, but BA-pretreated cells are highly migratory. I have shown that BA modifies the organization of diffuse actin and actin bundles. This modification probably occurs through controlling the expression of actin binding proteins such as alpha actinin (Ryan and Higgins, 1987). Answers to how BA affects actin need to be solved at the molecular level.

Actin bundles can change cell shape and regulate cell movement. I have shown in Chapter VI that the process of migration and invasion can change the morphology and amount of total F-actin, but only a combination of factors (substrate interactions and drug treatment) can account for the actin morphology observed in invasive and migratory cells. The interaction between drug treatment and the migratory process is important. For example, there was no significant difference

in total F-actin between untreated and BA-treated nonmigratory cells, but there was significantly more total F-actin in BA-treated cells than untreated migratory cells. However, the effect of drug interactions with the migratory or invasive processes on the F-actin does not tell the whole story. There were differences in actin morphology and total F-actin between untreated migratory and invasive cells. In addition, the affect of substrate interactions with drug treatment on F-actin morphology was observed in comparing noninvasive untreated and drug treated cells. Transformed motile cells, transformed nonmotile cells, and untransformed motile cells are interesting model systems to examine the mechanisms behind cellular motility. Drugs that affect cellular movement expand the model systems. The role of differentiating agents, such as DMSO and DMF, on motility would be interesting to investigate since they are known to change the morphology of tumor cells (Lampugnani et al., 1987; Dexter et al., 1974).

The Transwell system allows for the isolation of unique subpopulations of cells (nonmigratory/migratory and noninvasive/invasive) for further analysis. One question that was not addressed in my experiments was whether there was a quantifiable change in the nonactin cytoskeleton by BA or RA. There is, apparently, a role for microtubules as shown by TEM in the migration of untreated and drug treated cells through the pores, but quantification was not posssible. It is clear that the morphology of actin filaments is distinctive in subpopulations of untreated B16a cells. The actin morphology of migratory and invasive untreated cells is different from nonmigratory and noninvasive untreated cells and is not just a result of substrate

interactions.

Substrate interactions do have an effect on morphology, motility and metastasis of tumor cells (Nabi et al., 1990). In my study, there are differences in the actin morphology of nonmigratory and noninvasive untreated cells. The majority of F-actin filaments of noninvasive cells is confined to diffuse actin. In contrast, actin filaments (not diffuse actin) dominate the cytoplasm of untreated cells grown on uncoated polycarbonate membranes. The noninvasive and nonmigratory cells were similiar in that the majority of the cells grew in clusters.

Migration and cell shape are related to metastasis? For example, an increase in motility and lung colonization was found in B16F1 melanoma cells that were grown as spheroids on a nonadhesive substrate (Poly(HEMA)) (Raz and Ben-Ze'ev, 1983; Nabi et al., 1990). B16F1 cells that were grown on Poly(HEMA) and allowed to reattach to tissue culture treated plastic were found to have increased spreading characteristics compared to cells that were grown only on the tissue culture treated plastic. Increased spreading was correlated to increased motility (Nabi et al., 1990). This is interesting to note since I have noted increased spreading and migratory capability of BA-treated B16a cells (McGarvey and Persky, 1989). The molecular events that increased motility in Poly (HEMA) grown cells may be related to events that increase the migration of BA-treated cells. The actin morphology of noninvasive BA-pretreated B16a cells does not appear to be different from nonmigratory BA-pretreated cells. However, I have found by image analysis that there is an increase in actin 3, which represents the highest level of actin organization. This information is further

evidence that higher levels of organization of actin is correlated with decreased migration.

The Matrigel Invasion Assay

The Matrigel assay can be modified to further understand the invasion process. In this dissertation, a chemoattractant from NIH fibroblasts was added to the lower chamber of the Transwell chamber. The advantage of this modification is that the time period for the invasion assay was shortened and that an inhibition of proliferation can be separated from an inhibition in invasion. The addition of the chemoattractant increased the invasion rate of untreated cells, but likewise increased the invasion rate of BA-pretreated cells. Other modifications of the Matrigel assay have been done. For example, additional amounts of collagen type IV have been added to the Matrigel (Wang and Stearns, 1988). They found that invasion of tumor cells decreased through the modified Matrigel as compared to the invasion through Matrigel alone.

I have shown that cellular adhesion to Matrigel was enhanced by BA pretreatment. A major constituent of Matrigel is laminin. The interaction of tumor cells with laminin is important since BA has been shown to reduce the number of laminin receptors in a human pancreatic cell line (Bryant et al., 1986). However, laminin has also been shown to enhance the release of type IV collagenase (Turpeenniemi-Hujanen, 1986). The Transwell system lends itself to manipulation to further elucidate the factors involved in cellular migration and invasion. The bottom surfaces of polycarbonate membranes may be coated with various basement membrane proteins for haptotactic assays or with chemotactic

peptides. Likewise, proteins may be added to the lower chamber to increase migration and invasion.

A major concern about Matrigel for invasion assays is that it forms at varying concentrations a consistent uniform barrier. Scanning microscopy, transmission electron microscopy, and fluorescent diffusion studies have confirmed the integrity of the Matrigel barrier (Persky et al., 1990). Basement membrane products such as thrombospondin, which is not present in Matrigel is important to the invasion process (Roberts et al., 1987). Thrombospondin induced chemotactic and haptotactic responses in several human tumor cell lines (Roberts et al., 1987). A defined basement membrane product similiar to Matrigel, that is functionally analogous to the human amnion, which contains a basement membrane and a collagenous stroma, would be advantageous in tumor invasion studies.

It is clear from this dissertation that in vitro assays may not tell the whole story. My in vitro experiments evaluated two agents. Butyric acid increased the adhesion, migration and invasion of B16a cells. In contrast, the other agent, RA decreased migration and invasion. However, both agents inhibited experimental metastasis. The intricate nature of the metastatic cascade is not limited to tumor cell invasion or migration, but includes other complex interactions with the host. Liotta (1970) observed that only 0.1% of the cells injected into tail veins of mice produced metastatic foci. The tail vein protocol includes the steps of entrapment of cells in the lungs, extravasation, and growth of the foci, yet only a small number of cells survive. Since this time, the role of phenotypic selection of tumor cells in

metastasis has gained exceptance. Metastasis may be conceptually be thought of as a series of limiting steps or gates. These steps include lung entrapment, extravasation (which includes adhesion, invasion and migration), growth, and escape from the host defenses. Administration of BA may open the invasion gate, but close the proliferation gate allowing host defences to eradicate the tumor. Retinoic acid can have affect any point in the experimental metastatic assay, closing still tighter the various gates. In addition, various cell types including placental trophoblasts, which are neither tumorigenic nor metastatic, can migrate and invade through Matrigel (Librach et al., 1989). Although the Matrigel and migration assays are interesting reductionist model systems to study tumor cells, the importance of the in vivo metastasis assays (experimental and spontaneous) can not be overemphasized.

CHAPTER VIII

SUMMARY

The affect of two potential antitumor agents, butyric acid (BA) and all trans-retinoic acid (RA), on murine B16a melanoma cells was investigated in vitro and in vivo. Initial studies determined the cytostatic/cytotoxic concentrations of BA and RA by hemacytometer counts and by the trypan blue exclusion test. Cytostasis was confirmed by autoradiography and beta scintillation measurement of incorporation of either ^{3}H-thymidine or ^{3}H-Iodo-deoxyuridine.

The in vitro studies included both migration and invasion assays. For the migration assays, cells were plated onto 3 and 5 um diameter pore polycarbonate membranes. For the invasion assays, cells were plated onto recombinant basment membrane coated 8 um pore polycarbonate membranes. Migration and invasion rates were quantified by scanning electron microscopy. Cells were treated with the cytostatic concentrations of BA and RA or untreated for 72 hrs before the migration and invasion assay. Butyric acid or RA was again added at the time of plating of cells onto the membranes.

The cytoskeleton of untreated and drug-treated migratory and invasive cells was also investigated by transmission electron microscopy and fluorescent microscopy. Cytoskeletal changes were quantified by image analysis of F-actin that had been stained by Bodipy phallacidin.

The in vivo study used the experimental metastatic assay (tail vein inoculation). Butyric acid or RA containing pellets, as well as placebo pellets, were administered subcutaneously.

The major findings were:

1. The range of cytostatic/cytotoxic concentrations of RA were 1

$X\ 10^{-7}$ - $1\ X\ 10^{-6}$ / > $1\ X\ 10^{-5}$ M and of BA were .5 mM - 1.5 mM / 2.0 mM.

3. The chosen cytostatic concentration of BA (1.5 mM) decreased by 80% the incoporation of ^{3}H-thymidine.

4. The chosen cytostatic concentration of RA ($1\ X\ 10^{-7}$ M) decreased by 72.3% the incorporation of ^{3}H-IdUR.

5. The cytostatic concentration of BA decreased incorporation of ^{3}I-dUR within 24 hrs.

7. Butyric acid increased migration and invasion of B16a cells. Pretreatment with BA further increased migration or invasion.

9. Retinoic acid decreased migration and invasion of B16a cells.

10. Administration of BA and RA by subcutaneous pellet significantly decreased the number of lung nodules. In addition, administration of BA by subcutaneous pellet also decreased the incidence of lung nodule formation.

12. Both migratory and invasive cells formed pseudopods containing networks of cortical actin and parallel microtubules.

13. Migratory untreated cells had significantly less filamentous F-actin than migratory BA-pretreated cells and migratory RA-pretreated cells.

14. Invasive cells with or without drug treatment had similiar amounts of filamentous F-actin.

15. Untreated and drug-treated invasive and migratory cells were capable in varying degrees of bundling actin. This finding is important since it has been reported that invasive cells contained only diffuse actin.

17. Untreated/drug treated noninvasive and nonmigratory cells have more actin 3 (the highest level of actin organization) than untreated/drug treated invasive and migratory cells.

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APPENDIX I

The appendix is titled "Discussion with Reviewers" and has been published in the paper "The Effects of Butyric Acid and Retinoic Acid on *In Vitro* Migration by Murine Bl6a Melanoma Cells: A Quantitative Scanning Electron Microscopy Study." Scanning Microscopy, 3 (2): 591 - 604. The appendix contains material pertinent to Chapter IV.

DISCUSSION WITH REVIEWERS

R. Lotan: What is a diffusion chamber?

Authors: The diffusion chamber is a modification of the Membrane Invasion Culture System (MICS) chamber (Gehlsen et al., 1984; see References). Figure 13 is a 14 well MICS chamber. The diffusion chamber is similar to the MICS chamber except that the diffusion chamber has 45 wells. In the migration assays, complete medium with or without a drug is added to the lower wells. The polycarbonate membrane is placed between an upper and lower chamber, creating 45 separate upper and lower wells. Cells are then added in complete medium, with or without a drug, to the upper wells.

Figure 13: MEMBRANE INVASION CULTURE SYSTEM

(MICS; Pat Pending 84-027-1)

(A) Top Plate

(B) Bottom Plate

(C and E) Upper Wells

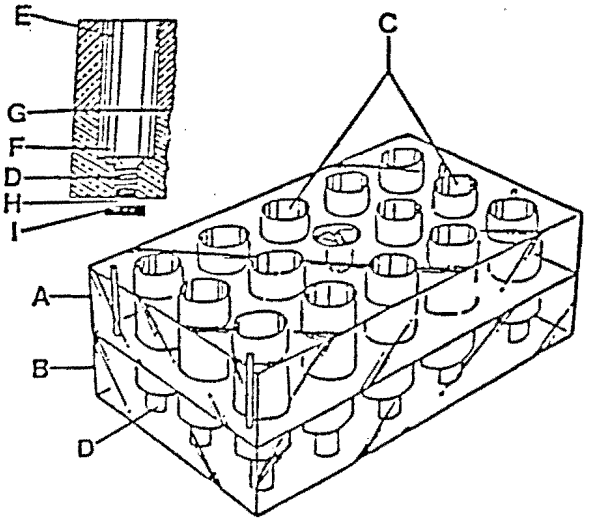
(D and F) Lower Wells

(G) Polycarbonate Membrane

(H) Washer

(I) Bolt

Redrawn with permission from Hendrix et al. (1985).



M.J.C. Hendrix: It is very difficult to derive significant statistical information from Table 4 when less than seven cells are presented?

Authors: As a purely statistical question, this is a valid criticism. Table 4 presents data obtained from 50 fields at 1700X. Data could have been presented at lower magnifications, e.g., 500X. At 500X, the exact same quantitated surface area would have provided 15 fields (1,700 divided by 500 = 3.4; 50 divided by 3.4 = 15). The mean number of cells for Table 4 would therefore be 3.4X greater. I chose to present the data at 1,700X because we could positively distinguish individual cells at higher magnification.

R.Lotan: It would have been interesting if a low metastatic variant had been used in the migration assay.

Authors: This is true, but the objectives of this project are to evaluate the effect of two drugs on a highly metastatic cell line. This project is part of an overall investigation that will evaluate the effect of RA and BA on viability, proliferation, migration, invasion, and metastasis. Since the human amnionic basement membrane (HABM) assay of Liotta et al. (New method for preparing large surfaces of intact human basement membrane for tumor invasion studies. Cancer Letters, 11:141-152, 1980) is being used as one of the invasion assays, and since the HABM assay is only valid for highly invasive cells (Welch, DR. 1986 Discussion of the suitability, availability and requirements for in vivo and in vitro models of metastasis. In: Cancer Metastasis, Experimental and Clinical Strategies. (eds). DR. Welch, BK. Bhuyan, and LA. Liotta. Alan R. Liss, Inc., N.Y., p. 135). Low metastatic cells were not used.

M.J.C. Hendrix: Is the rapid rate of biological degradation of retinoic acid by tumor cells similar for butyric acid? If not, what bearing would this information have on the data generated in this study?

Authors: To our knowledge the rate of biological degradation of BA by tumor cells has not been reported. The incorporation of tritiated RA by murine S91 melanoma cells has been reported to be rapid during the first 15 min. Tritiated RA is incorporated much more slowly after this initial 15 min time period (Lotan et al., Characterization of retinoic acid-induced alterations in the proliferation and differentiation of a murine and a human melanoma cell line in culture. Ann N.Y. Acad Sci 359: 150-170, 1981). In addition, S91 and B16 melanoma cells, when grown in 10^{-5} M RA for six days and then refed in RA-free medium, continued to proliferate at a reduced rate for 24-48 h. However, after 3 days of incubation in RA-free medium, the growth rate of previously RA-treated cells returned to that of the control cells (Lotan et al., Characterization of the inhibitory effects of retinoids on the vitro growth of two malignant murine melanomas. JNCI 60: 1035-1041, 1980). This reversible inhibition of growth of various tumor cell lines has been reported by others using BA (Prasad and Sinha, Effect of sodium butyrate on mammalian cells in culture: a review. In Vitro 12:125-135, 1976). I have observed reversible changes in morphology of RA and BA-treated B16a cells after removal of the drug from the medium. Therefore, despite a rapid incorporation and rapid degradation of RA (and possibly BA) these drugs remain effective in changing morphology and inhibiting growth beyond the 72 h experimental time period.

M.J.C. Hendrix: Was scanning electron microscopy the only technique used to quantitate the tumor cells on the polycarbonate membranes? If so, what precautions were taken to insure that the entire filter was examined with SEM? Weren't the samples quite large to handle?

Authors: Our intial attempts to quantitate cells by light microscopy were unsuccessful because we were not able to distinguish cells above, in, and below the membrane. Radioisotope labelling of cells was also attempted, but difficulties were encountered in removing BA-treated cells from the polycarbonate membranes. Even a 20 min trypsin/EDTA incubation failed to completely remove the BA-treated cells from the membrane. The entire membrane was observed by SEM. Care was taken to select 50 random fields to quanitate migration. Samples were not too large to handle. On the contrary the small size of the membranes permitted three complete Transwell membranes to be loaded at one time into the scanning scope.

M.J.C. Hendrix: Do the authors propose that SEM offers a universal quantitation approach for tumor cell invasion that supersedes currently available technology?

Authors: Scanning electron microscopy is an accurate and reproducible method for quantifying migration and invasion. SEM allows visualization of top and bottom membrane surfaces and permits observation of individual cells. SEM does not have the depth of field resolution problem that is associated with light microscopy. SEM is but one method to quantitate cell invasion and migration.

R. Lotan: Why is it probable that increasing the pore size from 3 um to 5 um allows "flattened" cells to migrate?

Authors: I have shown that the migration of early passage untreated B16a cells through 5 um diameter pore membranes is 66% greater than the migration through 3 um diameter pore membranes (Trials 1 and 3, Table 4). I have observed that cells on the top surface of the 3 um diameter pore membrane are heterogeneous in shape and contain flattened cells. In contrast, the heterogeneous population of cells on top of 5 um diameter pore membranes have few flattened cells. It appears that the majority of cells on the bottom surface of the 5 um pore membrane are flat in shape.

R. Lotan: It is possible to dehydrate tissues for SEM with alcohol instead of acetone that would have allowed you to critical point dry the specimens instead of air drying them. Also, the membranes can be cut out of the plastic well and processed separately.

Authors: Despite the cracking produced by air drying, problems were not encountered in quantitating cells. The cracking produced by air drying the filters can be reduced by using hexamethyldisilazane (Nation et al. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. Stain Tech. 58:347-351, 1983). It is possible to dehydrate with alcohol instead of acetone before critical point drying. Our electron microscopy facility does not have a suitable drying agent, such as Freon 113, for critical point drying alcohol dehydrated specimens. The membranes were processed in Transwell chambers to facilitate ease of handling. Cutting out the membranes prior to alcohol dehydration causes the membranes to roll up.

Reviewer #4: Is the rate of proliferation the same between cells grown

on polycarbonate membranes as cells grown on tissue culture treated plastic?

Authors: Yes. I have previously determined that there is no significant difference between B16a cell proliferation rates for cells grown on plastic dishes versus polycarbonate membranes. I quantified proliferation by plating cells into Transwell chambers (50,000 cells/well) and into a 24 well plate (Corning) (50,000 cells/well). Each experiment was done in triplicate and the number of cells determined after 72 h by hemacytometer count.

Reviewer #4: Why did the authors use two different radioisotopes to determine proliferation rates?

Authors: After completing the BA experiments with ^{3}H-thymidine, we changed radioisotopes to ^{3}H-IdUR. The irreversible binding property of ^{3}H-IdUR makes it a better radiolabel than ^{3}H-thymidine (Fidler, I. Metastasis: quantitative analysis of distribution and fate of tumor emboli labelled with ^{125}I-5-Iodo-2'-deoxyuridine. JNCI, 45:773-782, 1971). Since BA had already been shown to produce a significant inhibition of ^{3}H-thymidine incorporation, we thought it would not be necessary to repeat the BA experiments using ^{3}H-IdUR.

Since the publication of this manuscript, the BA experiments were performed using ^{3}HIdUR. Butyric acid inhibited the incorporation of ^{3}HIdUR by 76% after 18 h incubation (54 h preincubation with BA) and 84% after 48 h incubation (24 h preincubation with BA).

Reviewer #4: The methodology of choice for determining cytotoxic/cytotstatic effects of drugs is the double-label growth assay. Why did the authors not use this technique?

Authors: I agree that the double label growth assay is a preferred method to quantitate viablity and proliferation in drug studies. Although the trypan blue exclusion method is less sensitive, it did provide cytotoxic/cytostatic data that was statistically significant.

APPENDIX II

This appendix is titled "Transmission Electron Microscopy of the Cytoskeleton of Migratory and Invasive Cells" and has been published under the same name in the Journal of Electron Microscopy Technique, 13(3): 272 - 273. This appendix describes the methodology used to evaluate the cytoskeleton of migratory and invasive cells by transmission electron microscopy. This methodology is pertinent to Chapter VI.

INTRODUCTION. The recent development of the Transwell cell culture chamber insert (Costar Co., Cambridge, MA) permits analysis of cellular migration and invasion. Transwell inserts contain a porous polycarbonate membrane and fit into 6 and 24 well cluster plates. Although cellular migration and invasion through polycarbonate membranes have been quantitated by routine LM, SEM and radiolabel techniques, few reports have focused on the cytoskeleton of cells in the process of moving through pores. This communication reports a simple methodology to evaluate the cytoskeleton of both migratory and invasive tumor cells within 5 um and 8 um diameter pore Transwell polycarbonate membranes.

MATERIALS AND METHODS. Murine B16a melanoma cells were cultured in Eagle's medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and l-glutamine supplemented with sodium pyruvate (Sigma, St. Louis, MO), 10% heat inactivated fetal calf serum (FCS) (Gibco), 1% penicillin G-streptomycin sulfate-amphotericin B (Fungizone) (Gibco), MEM non essential amino acids (Gibco), sodium bicarbonate (Sigma), and Hepes (Sigma). Cells were treated with sodium butyrate acid (BA) (JT Baker, Phillipsburg, NJ) for 3 days before plating onto the Transwell membranes. Transwell membranes were uncoated for migration assays (McGarvey and Persky, 1989) and coated with Matrigel (Collaborative Research, Cambridge, MA) for invasion assays (Repesh, 1989).

Transmission electron microscopy (TEM) followed the method of McDonald (1984) in order to preserve microfilaments. Membranes were fixed for 30 min at 37^{o}C with 2% glutaraldehyde in 50 mM cacodylate

buffer (pH 7.4) containing 5 mM $CaCl_2$, rinsed in buffer (3X) for 5 min at 37°C and incubated for 30 min in buffer containing 0.8% $K_3Fe(CN)_6$. Membranes were then incubated for 30 min in buffer containing 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$, rinsed in buffer, rinsed in distilled water (3X), stained en bloc with 2% uranyl acetate (distilled water) for 60 to 120 min, rinsed in distilled water (3X) and dehydrated in ethanol. Membranes were infiltrated into resin (Epon-Araldite), cut out of the Transwell inset, and polymerized in blocks at 60°C for 3 days. Thin sections were cut, stained in 1% uranyl acetate (in 70% methanol) and lead citrate, and viewed with an Hitachi H-600 microscope at 75 kV.

RESULTS AND DISCUSSION. This investigation reports a simple method to prepare and analyze by TEM the cytoskeleton of migratory and invasive cells within a polycarbonate membrane. Figure 1 demonstrates a migratroy cell be seen extending two large pseudopods into two 5 um pores. Anchoring of the cell to the polycarbonate membrane appears to take place both within the pores as well as above and below the membrane (arrows). Higher magnification (Fig. 2) reveals an extensive parallel arrangement of the cytoskeleton within one of the large pseudopods. The cell border displays bundles of cortical actin. Cortical actin has been reported to have a major role in cell motility and migration (Hay, 1985). It is obvious that the actin preservation is excellent.

Butyric acid (BA) was used in this study at a concentration of 1.5 mM. This concentration has been shown to be noncytotoxic, to significantly increase cellular migration, and to induce changes in the

cytoskeleton of B16a cells (McGarvey and Persky, 1989). The arrangement of the cytoskeleton in the large pseudopod (Fig 2) may not only be indicitive of a migrating cell but also of BA treatment.

The Transwell insert is an important new tool to analyze cellular migration and invasion. The polycarbonate membrane of the Transwell insert was uncoated for migration and coated with a reconstituted basement membrane, Matrigel, for invasion. Figure 3 depicts a BA-treated cell on top of the Matrigel. A portion of this cell and possibly another cell have extended into an 8 um pore by digesting the Matrigel (arrow). Higher magnification of this area (Fig. 4) reveals a cytoplasm without the extensive cortical actin and other cytoskeletal filaments seen in the migratory cell. Areas of contact with the Matrigel appear to contain actin (arrows). Kramer et al. (1989) have reported fibrosarcoma cells, which invaded Matrigel, to have (1) formed pseudopodia which contained organized microfilaments and (2) have cleared the matrix adjacent to the pseudopodia. While we did not observe bundles of microfilaments, matrix clearing (mc) was evident around the pseudopodia.

The ease of handling the Transwell insert is a major advantage for microscopy. Membranes can be processed for TEM in the Transwell inserts until embedding in the plastic. Together, the Transwell insert and Matrigel lend themselves to techiques such as immunogold to locate and identify cytoskeletal proteins and proteolytic enzymes in cells that are <u>within</u> the pores of the polycarbonate membrane.

FIGURE 1.

A migrating BA-treated B16a cell cultured on a 5 um diameter pore polycarbonate membrane (pc). A large pseudopod extends the length of a pore with a smaller projection within a second pore (arrows). Bar = 2 um.

FIGURE 2.

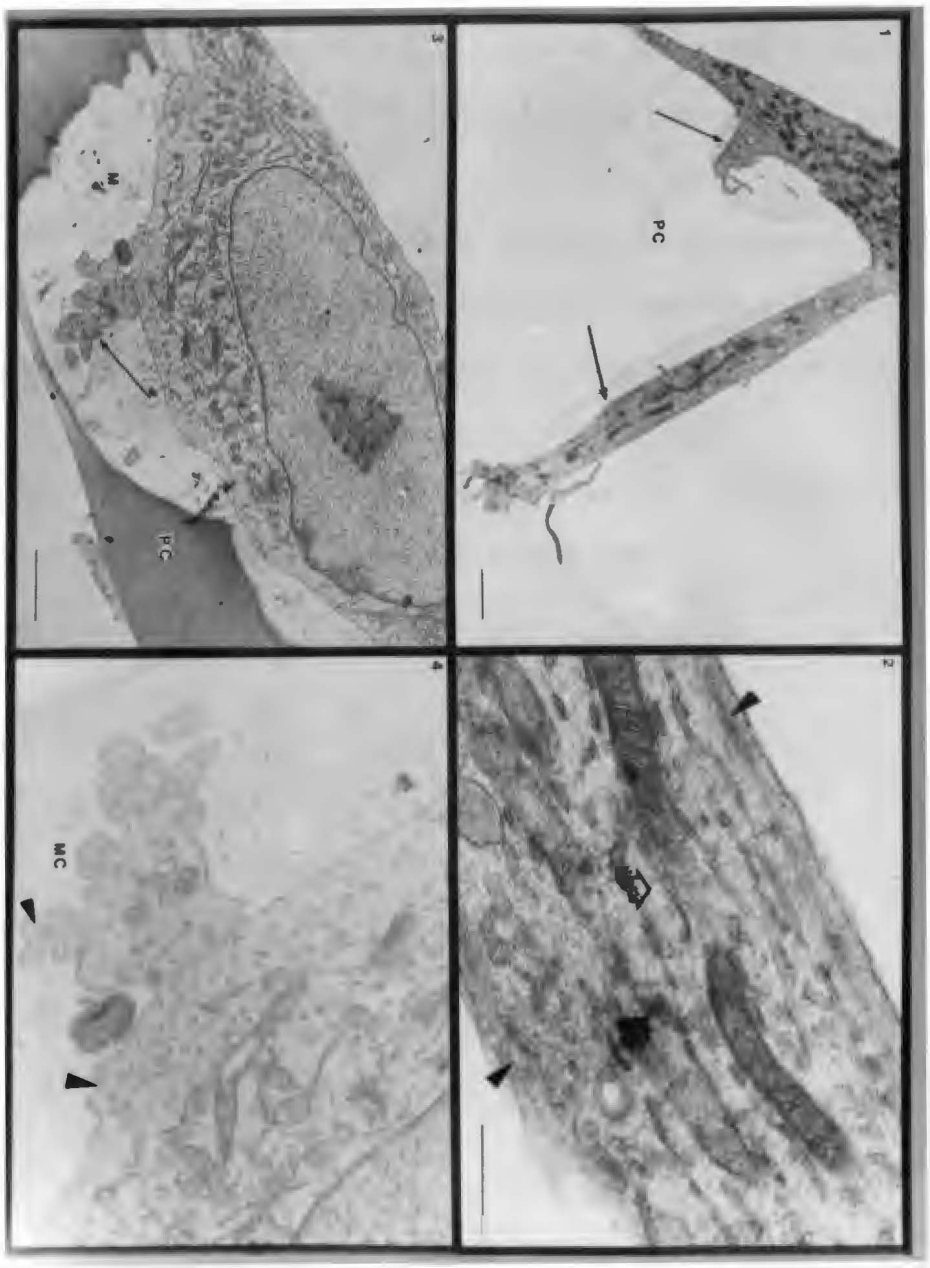
Higher magnification of the large pseudopod of Fig 1. Note the extensive actin (a) , intermediate filament () , and microtubule () system. Bar = 1 um.

FIGURE 3.

An invasive BA-treated B16a cell cultured on a Matrigel coated (m) 8 um diameter pore polycarbonate membrane (pc). A projection of this cell or possibly another cell (arrow) is found below the Matrigel barrier. Bar = 2 um.

FIGURE 4.

A higher magnification of the projection of Fig 3 with some cortical actin (a) as well as a clear Matrigel clear zone (mc). Bar = 0.5 um.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Repesh, L.A. (1989) A new in vitro assay for quantitating tumor cell invasion. Invasion Metastasis, 9: 192-208.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 7, 1990
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